Antagonism of Roseobacter clade bacteria against pathogenic bacteria

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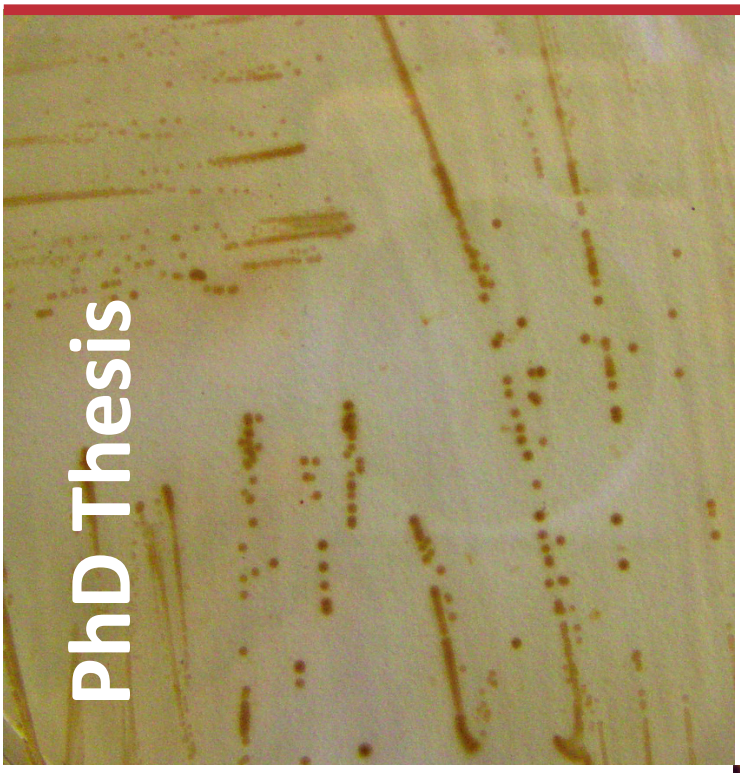
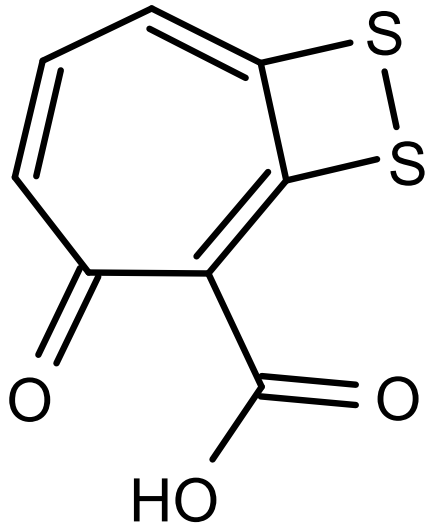
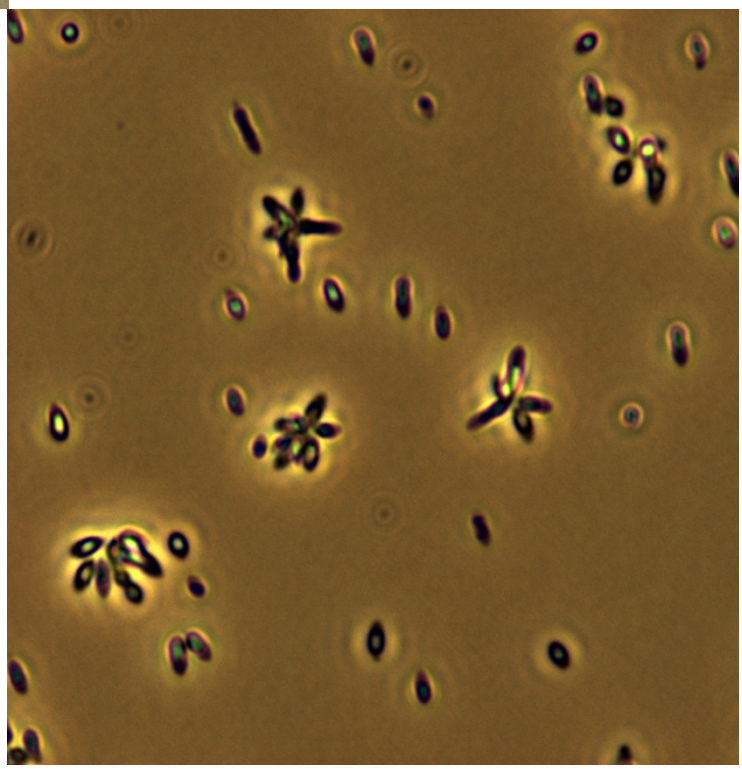




By Cisse Hedegaard Porsby



DTU Food
National Food Institute

Antagonism of *Roseobacter* clade bacteria against pathogenic bacteria

PhD thesis

by

Cisse Hedegaard Porsby

2010

Technical University of Denmark

National Food Institute

Division of Industrial Food Research

Front page photos:

Upper left: *Phaeobacter* strain M23-3.1 on marine agar plate

Upper right: Molecule structure of tropodithietic acid

Bottom right: *Phaeobacter* strain 27-4 grown in marine broth at stagnant conditions

Preface

The work presented in this thesis is the outcome of my PhD study at the Technical University of Denmark (DTU). The project was financed by the Research Council for Technology and Production (project 09-061086).

From January 1st, 2007 to September 13th, 2010 I have been enrolled at The National Institute of Aquatic Resources, Department of Seafood Research which later was merged into DTU, National Food Institute, Division of Industrial Food Research. (DTU Food). I have been on maternity leave during that time period.

The work has primarily been carried out at DTU Food, but included a 12 weeks stay with Professor Laura Piddock and Dr. Mark Webber, Antimicrobial Agents Research Group, School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham.

Professor Lone Gram (DTU Food) has supervised this project.

Cisse Hedegaard Porsby

Kgs. Lyngby, September 2010

This thesis is based on the following two papers:

Paper 1:

Cisse Hedegaard Porsby, Kristian Fog Nielsen and Lone Gram. (2008). *Phaeobacter* and *Ruegeria* species of the *Roseobacter* clade colonize different niches in a Danish turbot (*Scophthalmus maximus*) rearing farm and antagonize turbot larval pathogens under different growth conditions. *Applied and Environmental Microbiology* **74:**7356-7354

Paper 2:

Cisse Hedegaard Porsby, Mark A. Webber, Kristian Fog Nielsen, Laura J. V. Piddock, Lone Gram. Resistance and tolerance to tropodithietic acid, an antimicrobial in aquaculture, is hard to select. *Antimicrobial Agents and Chemotherapy:* ***Submitted***

Furthermore, I have contributed to the following articles during my Ph.D. study:

Poul D'Alvise, Jette Melchiorsen, **Cisse Hedegaard Porsby**, Kristian Fog Nielsen and Lone Gram (2010). Inactivation of *Vibrio anguillarum* by attached and planktonic *Roseobacter* cells. *Applied and Environmental Microbiology* **76:**2366-2370

Lone Gram, **Cisse Hedegaard Porsby**, Mette Jensen, Jette Melchiorsen and Kristian Fog Nielsen. A cosmopolitan bacterium: comparing phylogenetic and phenotypic homogeneity in a global collection of *Ruegeria mobilis* to other *Roseobacter* clade strains. *Under revision*.

Poster presentation at international meetings:

Cisse Hedegaard Porsby, Dan Erik Stenvall, Jesper Bartholin Bruhn and Lone Gram (2008). Culturable antagonistic *Roseobacter* clade strains isolated from turbot farms are genetically distinct from strains isolated from oceanic environments. '108th General Meeting' American Society for Microbiology in Boston, Massachusetts 1^{st}-6^{th} Juni 2008.

Summary

Today antibiotics are used to restrain bacterial diseases in aquaculture, however, alternative strategies for bacterial disease control are needed due to the risk of resistance development and transfer of resistance to other bacteria e.g. human pathogens. Probiotics, which are "*live organisms which when administered in adequate amounts confer a health benefit on the host*" (FAO/WHO, 2001), can probably be used in e.g. fish larvae cultures. *Phaeobacter* and *Ruegeria* species, which belong to the marine *Roseobacter* clade, have been suggested as probiotic organisms in aquaculture. They inhibit or kill fish pathogenic bacteria *in vitro* and are capable of reducing mortality of fish larvae infected with fish pathogenic bacteria. It is hypothesized that this effect is caused by production of the secondary metabolite tropodithietic acid (TDA).

The purpose of the present PhD study was to investigate the occurrence of antagonistic *Roseobacter* clade strains in aquaculture and to determine the growth conditions under which these strains produce TDA. Also, the thesis has evaluated the risk/likelihood of development of resistance and tolerance to TDA.

This study showed that a Danish turbot larvae rearing unit was colonized with roseobacters that inhibited the fish pathogenic bacteria *Vibrio anguillarum* strain 90-11-287. Strains isolated from the production site (e.g. fish tanks) were by phenotypic tests and 16S rRNA phylogeny identified as *Phaeobacter inhibens* and *Phaeobacter gallaeciensis*-like, whereas strains from algae culture were identical to *Ruegeria mobilis*. *Phaeobacter* and *Ruegeria* strain have previously been shown to inhabit Spanish turbot larvae rearing units. These Spanish strains were phenotypically and by 16S rRNA gene comparisons highly similar to the Danish strains isolated in this study. Despite the very different water sources in the two aquaculture settings (the Galician Atlantic Ocean versus the Danish fjord Limfjorden) they are both colonized with an antagonistic *Phaeobacter* / *Ruegeria* population.

All the *Phaeobacter* and *Ruegeria* strains isolated from the Danish turbot larvae rearing unit produced TDA at stagnant growth conditions and this phenotype co-occurred with the ability to inhibit *V. anguillarum* strain 90-11-287, the ability to grow in rosette-like structures and production of brown pigment. All the *Phaeobacter* strains also displayed these phenotypes when grown at shaking conditions, whereas this was not the case for the *Ruegeria* strains. The only exception from this pattern is strain 27-4 which is identified as *Phaeobacter*, but behaves like a *Ruegeria*.

Many attempts were tried in order to generate TDA resistant mutants, however, no mutants were isolated. It is thought that TDA has multiple target sites in a bacterial cell, and that resistance

requires a multitude of mutations in the cell that are unlikely to co-occur. After long-term exposure to subinhibitory concentrations of TDA, strains with a slightly increased tolerance to TDA were selected, but this tolerance was abolished after one passage in media without the antimicrobial agent. In this thesis it is hypothesized that this transient tolerance is due to a phenotypic switch which can be rapidly reverted, and it can be caused by e.g. altered gene expression.

This study showed that TDA is bactericidal against both Gram-positive and Gram-negative bacteria, with the Gram-positives being most susceptible. Neither efflux pumps nor porins are involved in the innate resistance of Gram-negative bacteria. TDA is active against both growing and non-growing *Salmonella* Typhimurium and *Staphylococcus aureus* cells, and no visible damage to *Escherichia coli* cells was observed after treatment with TDA. It is in this thesis hypothesized that TDA does not have to enter the interior of a bacterial cell to reach its target, and the cell envelope is suggested to contain the target site(s). This is in agreement with the biosensor results that also identify the cell envelope and possibly the peptidoglycan structure as target.

This thesis concludes that antagonistic *Phaeobacter* and *Ruegeria* strains colonize turbot larvae rearing units using different water sources. Production of TDA among these strains is highly growth dependent. The fact that *Phaeobacter* strains produce TDA at more versatile conditions make them promising probiotic candidates in aquaculture as stagnant conditions rarely are found in a fish tank. Furthermore, the fact that resistance to TDA is difficult to provoke, and that the tolerant phenotype seen are highly unstable makes TDA producing bacteria interesting for control of bacterial diseases in aquaculture. Also, TDA could have potential as antibiotic for treating bacterial infections in e.g. humans, however, further studies are needed to evaluate this potential.

Resumé (in Danish)

I dag bruges antibiotika til at kontrollere bakterielle sygdomme i akvakultur. Der er et stigende behov for alternative kontrolmetoder pga. risikoen for udvikling af antibiotikaresistens og overførsel af resistens til andre bakterier fx humanpatogene bakterier. Probiotika, hvilket er ”*levende organismer, der når de bliver administreret i passende mængder, har en fordelagtig effekt på værtens helbred*” (FAO/WHO, 2001) kan sandsynligvis bruges til fx kulturer af fiskelarver. *Phaeobacter* og *Ruegeria* arter, der tilhører den marine *Roseobacter* gruppe, er blevet forslået som probiotiske organismer i akvakultur. De hæmmer og dræber fiskepatogene bakterier *in vitro*, og de kan reducere dødeligheden blandt fiskelarver inficeret med fiskepatogene bakterier. Det er forslået, at denne effekt skyldes produktion af den sekundære metabolit ”tropodithietic acid” (TDA).

Formålet med dette ph.d. studie har været at undersøge forekomsten af antagonistiske bakterier tilhørende *Roseobacter* gruppen i akvakultur samt at bestemme under hvilke vækstforhold disse stammer danner TDA.

Arbejde i dette ph.d. studie har vist, at et dansk pighvar opdræt var koloniseret med roseobactere bakterier, der hæmmede den fiskepatogene bakterie *Vibrio anguillarum* stamme 90-11-287. Stammer isoleret fra produktionen (fx fisketanke) blev vha. fænotypiske tests og 16S rRNA fylogeni identificeret som *Phaeobacter inhibens* og *Phaeobacter gallaeciensis*-lignende, mens stammer isoleret fra algekulturer var identiske med *Ruegeria mobilis*. *Phaeobacter* og *Ruegeria* stammer er tidligere påvist at kolonisere spanske pighvar opdræt. De spanske stammer viste stor lighed fænotypisk og ved 16S rRNA fylogeni til de danske stammer. Til trods for der bruges forskelligt vand i de to opdræt (galisisk Atlanterhav versus den danske fjord Limfjorden) er de begge koloniseret med en antagonistisk *Phaeobacter* / *Ruegeria* population.

Alle *Phaeobacter* og *Ruegeria* stammer isoleret fra det danske pighvar opdræt producerede TDA under stillestående vækstforhold, og denne fænotype forekom samtidig med evnen til at hæmme *V. anguillarum* stamme 90-11-287, evnen til at vokse i roset-lignende strukturer og produktion af brunt pigment. Alle danske *Phaeobacter* stammer udtrykte også disse fænotyper under rystede vækstforhold, mens dette ikke var tilfældet for *Ruegeria* stammerne. Den eneste undtagelse fra dette mønster er stamme 27-4, som er identificeret til at være en *Phaeobacter*, men opfører sig som en *Ruegeria*.

Det blev udført mange forsøg på at lave TDA resistente mutanter, men der blev ikke isoleret nogle mutanter. Det er forslået at TDA har mange ’target sites’ i bakterieceller, hvorfor resistens kræver adskillige mutationer i cellen, hvilket er utænkeligt at ville finde sted. Stammer med svagt forøget

tolerance overfor TDA blev fundet efter lang tids påvirkning med koncentrationer af TDA, der ikke hæmmer væksten af bakterien. Denne tolerance forsvandt efter en passage i medie uden TDA. I denne afhandling foreslås det, at denne midlertidige tolerance forårsages af en fænotypisk forandring, der hurtigt kan ændres tilbage. Dette kan skyldes fx ændring i genekspression.

Dette ph.d studie har vist at TDA er baktericidal mod både Gram-positive og Gram-negative bakterier, dog er de Gram-positive mest følsomme overfor TDA. Hverken efflux pumper eller poriner er involverede i den medfødte resistens hos Gram-negative bakterier. TDA er virksomt mod både *Salmonella* Typhimurium og *Staphylococcus aureus* celler der vokser og ikke vokser, og der blev ikke observeret nogen synlige skader på *Escherichia coli* celler behandlet TDA. Det foreslås i denne afhandling at TDA ikke behøver at trænge ind i cellen for at nå sit target, og at cellevæggen eller cellemembranen indeholder target site(s). Disse to strukturer blev også identificeret vha. biosensorer og mere specifikt peptidoglykan-laget.

Det konkluderes i denne afhandling at antagonistiske *Phaeobacter* og *Ruegeria* stammer koloniserer pighvar opdræt, der anvender forskellig vand forsyning. Produktion af TDA blandt disse stammer er afhængigt af vækstforholdene, og det faktum at *Phaeobacter* stammer producerer TDA under mere alsidige forhold gør dem til lovende probiotiske kandidater i akvakultur, idet stillestående forhold er sjældne i fiskeopdræt. Den kendsgerning, at det er svært at fremprovokere resistens overfor TDA samt at den tolerante fænotype er meget ustabil, gør TDA producerende bakterier interessante til kontrol af bakterielle fiskesygdomme i akvakultur. Desuden kan TDA have potentiale som antibiotika til behandling af bakterielle infektioner i fx mennesker, men yderligere studier er nødvendige for at afdække dette potentiale.

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1 Introduction

Fish is a very important food source and provides more than 2.9 billion people with at least 15 percent of their average per capita animal protein intake. Actually, the majority (77%) of world total fish production was in 2006 used for direct human consumption (FAO/WHO, 2009). During the last decades, wild capture fisheries have stagnated whereas aquaculture, which is rearing of live aquatic animals, has grown rapidly (Figure 1.1). This makes aquaculture both an essential food source but also an important industry worldwide.

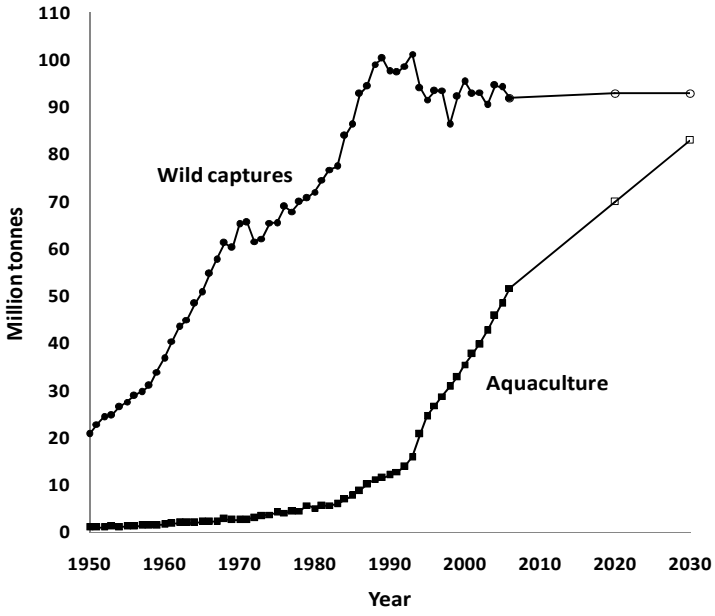

Figure 1.1 World fish production divided into wild captures and fish raised in aquaculture (closed symbols) and estimates for the following decades (open symbols) (FAO/WHO, 2009)

Fish reared in aquaculture are exposed to a variety of both physiological and social stresses, which can make them more vulnerable with regard to spread of infectious diseases. As this production form is very intense, it facilitates spreading of pathogenic organisms between the individuals. Diseases can be caused by parasites, viruses, fungi and bacteria but this thesis will only focus on bacterial infections. Despite successful vaccination programs, it is not un-common in aquaculture to control pathogenic bacteria with antibiotics. However, with this comes the risk of antibiotic residues in fish food products but also the fear of emergence of antibiotic resistant bacteria (Seyfried *et al.,* 2010). This has some serious side effects as antibiotics are no longer effective against the pathogens (Karunasagar *et al.,* 1994; Tendencia & de la Pena, 2001), which can lead to treatment failure. Furthermore, resistance genes can be transferred between bacteria including human pathogenic bacteria as recipients (Son *et al.,* 1997; Schwarz *et al.,* 2001). Therefore, antibiotic resistance originating from aquaculture can potentially be transferred to human pathogens and in worst case scenario cause disease treatment failure in humans. The European Union and USA have banned or

restricted the use of several antibiotics in animal production because of that (Kesarcodi-Watson *et al.,* 2008). As a result, there is an urgent need for alternative methods to control bacterial infections.

Vaccination of fish has indeed proven to be an efficient alternative for controlling bacterial infections. A good example is Norway where the use of antibiotic decreased from approx. 50 metric tonnes per year in 1987 to 746.5 kg in 1997, and at the same time aquaculture production increased from 5 x 10^4 to 3.5 x 10^5 metric tones (Verschuere *et al.,* 2000b). This is ascribed to introduction of efficient vaccines. However, during hatching and in the larval stage the size of the fish and the slow development of the immune system make it difficult to use vaccines. Also, invertebrates such as crustaceans and mollusks, which lack acquired immunity, cannot successfully be vaccinated.

An alternative disease control strategy, which seems very promising in aquaculture, is the use probiotics. Probiotics are "*live organisms which when administered in adequate amounts confer a health benefit on the host*" (FAO/WHO, 2001). This approach is promising in several cases including those where vaccines are not an option. Many bacteria have been tested for their *in vitro* antagonistic effect against fish pathogenic bacteria and some of the potential probionts have also been tested in *in vivo* challenge trails (Ringø & Vadstein, 1998; Huys *et al.,* 2001; Vaseeharan & Ramasamy, 2003; Gullian *et al.,* 2004; Planas *et al.,* 2006). One group of bacteria that is receiving increasing attention in the marine aquaculture area is the so-called *Roseobacter* clade.

Bacteria belonging to the *Roseobacter* clade are commonly found in marine environments such as ocean surface water but they are also associated with algae and colonize marine surfaces (Dang & Lovell, 2002a; Buchan *et al.,* 2005). *Phaeobacter* spp. and *Ruegeria mobilis*, which belong to the *Roseobacter* clade, are commonly found in marine larval rearing systems (Ruiz-Ponte *et al.,* 1998; Hjelm *et al.,* 2004b; Porsby *et al.,* 2008; Prado *et al.,* 2009). *Phaeobacter gallaciencis, Ph. inhibens* and *Ruegeria mobilis* are able to inhibit growth of or kill a variety fish pathogenic bacteria (Ruiz-Ponte *et al.,* 1999; D'Alvise *et al.,* 2010) but they are not lethal to egg yolk sac turbot larvae (Hjelm *et al.,* 2004a). The antagonistic effect against other pathogenic bacteria is likely to be due to production of the secondary metabolite tropodithietic acid (TDA).

It is hypothesize that production of TDA is an important competitive phenotype for bacteria such as *Ph. gallaeciensis*, *Ph. inhibens* and *R. mobilis* both in their natural oceanic niches but also in aquaculture settings. However, if they are to be used as probiotics in aquaculture, the microbiota in the fish tank probably will be exposed to TDA. Therefore, this thesis focuses on the TDA production in *Ph. gallaeciensis*, *Ph. inhibens* and *R. mobilis* and the effect of TDA on other bacteria. In the latter area, we focus specifically on potential resistance or adaptation in target bacteria.

The purpose of the present PhD study has been to investigate occurrence of antagonistic *Roseobacter* clade strains in aquaculture and to determine the growth conditions under which these strains produce TDA (paper 1). Also, the thesis has evaluated the risk/likelihood of development of resistance and tolerance to TDA (paper 2). Work addressing TDA mechanism-of-action (using gene expression analyses in target bacteria) is in progress but has unfortunately not been finished before deadline.

This thesis consists of an overview section and two papers/manuscripts. The overview section describes probiotic organisms, their possible mechanism-of-actions and potential use in aquaculture (chapter 2). It also introduces the *Roseobacter* clade (chapter 3) and it describes production of TDA especially by *Phaeobacter* and *Ruegeria* stains (chapter 4). Finally, the effect of antimicrobials on other bacteria with regard to targets in the bacterial cell and resistance mechanisms are described (chapter 5). The experimental work and the results obtained during the Ph.D. study are described in two papers/manuscripts, however, results will also be presented in the overview section when appropriate.

2 Probiotic in aquaculture

The first discovery of probiotic organisms was reported in 1908 where Elie Metchnikoff observed that a large number of people in Bulgaria became more than 100-years. The author linked this observation to large consumptions of yoghurt and afterwards isolated bacteria from the yoghurt. Metchnikoff concluded that the bacteria were the cause of a health promoting effect (referred in (Gillor *et al.,* 2008)). During recent years, both scientists, companies and the general public have turned attention to the concept of probiotics.

The term "probiotic" literally means "for life" and originates from combining the Latin word *pro* (for) and the Greek word *bios* (life) (Zivkovic, 1999). It was introduced in the 1970s but the first widely accepted definition of probiotics for warm-blooded animals was *"a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance"* (Fuller, 1989). This definition associates the probiont with feed, but in aquaculture the surrounding environment (e.g. the culture water) is also an important medium for exposing the fish to the probiont (Gomez-Gil *et al.,* 2000). Therefore, Gatesoupe (1999) modified it to *"microbial cells that are administered in such a way as to enter the gastrointestinal (GI) tract and to be kept alive, with the aim of improving health"*. So far it has been assumed in the definition that the probiont should exerts its effect on the GI tract, however, in aquaculture the skin and the gills of a fish is just as important niches for invasion and proliferation of pathogenic bacteria and the probiotic organism can just as well be applied through those routes (Gram *et al.,* 2005). Consequently, Gram *et al.* (1999) broadened up the definition to *"a live microbial supplement which beneficially affects the host animal by improving its microbial balance"*. However, "*microbial balance*" is not very well described. Thus, the definition on probiotics used in this thesis is *"live organisms which when administered in adequate amounts confer a health benefit on the host"* (FAO/WHO, 2001). One may note that this FAO/WHO definition broadens the probiotic concept for all hosts, also warm-blooded animals. Adding beneficial microorganism to the water in aquaculture does on one hand resemble plant biocontrol as the microorganisms exert their effect on the outside (the skin and in the gills). On the other hand, it is very well possible that some are ingested and may act via the GI tract.

The definition of probiotic organisms from FAO/WHO states that it should have *"a health benefit on the host"*. Gram & Ringø (2005) discuss that even though alterations in microbial composition may affect survival of fish, this is not necessarily a prerequisite for a successful probiotic culture. The authors proposed that the effect of a probiotic organism should be measured as *"its ability to decrease frequency of disease and/or increase survival from lethal diseases"*.

2.1 Mechanism-of-action of probiotic cultures

The mechanism-of-action causing the effect of a probiotic bacterium is rarely known. Most often, a correlation is made between the *in vitro* observations that a pathogenic bacterium is inhibited and the *in vivo* disease reducing effect of the same potential probiotic bacterium (Verschuere *et al.,* 2000b). A probiotic organism can most likely exert its effect through more than one mechanism-of-action resulting in a collective effect on the host.

2.1.1 Antagonistic compounds

In this thesis, antagonistic compounds are defined as a chemical compounds produced by a probiotic organism and it is either bactericidal or bacteriostatic against other microorganisms. If bacteria producing such compounds are present in the intestine, on surfaces of the host or in the culture water, where the host is farmed, then it is believed to either prevent proliferation of pathogenic bacteria or to kill them. Examples of general antagonistic compounds are antibiotics, bacteriocins, siderophores, lysozymes, proteases, hydrogen peroxide, ammonia and diacetyl but also production of organic acids, which alters the pH value, is considered an important factor.

There are numerous studies showing that different bacteria can antagonize aquaculture pathogens *in vitro* (Westerdahl *et al.,* 1991; Bergh, 1995; Sugita *et al.,* 1997; Jorquera *et al.,* 2000; Longeon *et al.,* 2004; Bruhn *et al.,* 2005; Jayaprakash *et al.,* 2005; Rattanachuay *et al.,* 2010; Bjornsdottir *et al.,* 2010), however, as shown by Gram *et al.* (2001) this is no guarantee that the probiotic organism will exert a similar effect *in vivo* (Figure 2.1).

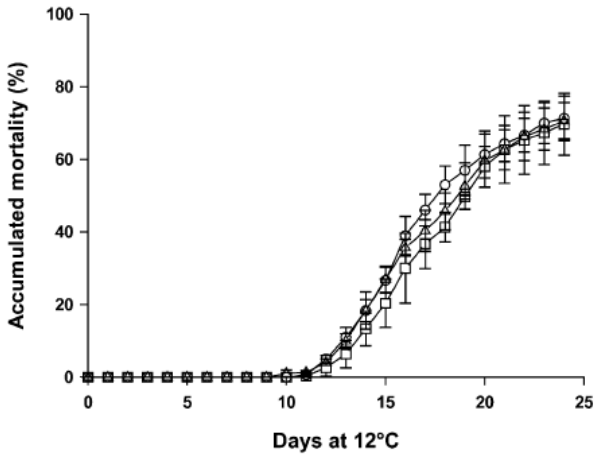

Figure 2.1 Accumulated mortality of Atlantic salmon (*Salmo salara* L.) infected with *Aeromonas salmonicida* with and without treatment (△) with *Pseudomonas fluorescens* strain AH2 at two levels: 0 – 10^5 (□) and 10^3 – 10^5 (○) cfu/ml, respectively. Fish of 15 – 20 gram sizes were kept in fresh water at 12°C with a flow of 0.8 l/kg fish per minute. Infection was done by 10% cohabitants and all treatments were carried out in triplicates. Error bars are 95% confidence limits (Gram *et al.,* 2001)

A few *in vivo* studies have suggested that the probiotic effect is caused by production of antagonistic compounds but the compound is not always identified (Table 2.1). Most of the experiments are conducted on shellfish larvae. It has never been shown in *in vivo* experiments, e.g. by measuring the concentration of the antagonistic compound, that the observed probiotic effect is

caused by the compound of interest. It is suggested in this thesis that using a mutant, which does not have the ability to produce the antagonistic compound, as a control in an *in vivo* infection study could indirectly show if the probiotic effect is due to that phenotype.

Table 2.1 Possible probiotic bacteria producing antagonistic compounds tested in *in vivo* studies

Producer strain	Pathogen inhibited	Possible compound	Probiotic against	Reference
Unidentified strain PM-4	*Vibrio* ssp.	Unknown	Shrimp larvae (*Penaeus monodon*)	(Maeda & Liao, 1992); (Maeda, 1994)
Vibrio sp.	*Vibrio anguillarum* related	Unknown	Scallop larvae (*Argopecten purpuratus*)	(Riquelme *et al.,* 1997)
Aeromonas media strain A199	*Vibrio tubiashii*	2,3-benzopyrrole	Pacific oyster larvae (*Crassostrea gigas*)	(Gibson *et al.,* 1998); (Lategan *et al.,* 2006)
Roseobacter strain BS107	*Vibrio anguillarum*	Proteinaceous, heat stable	Scallop larvae (P*ecten maximus*)	(Ruiz-Ponte *et al.,* 1999)
Roseobacter strain 27-4	*Vibrio anguillarum*	Tropodithietic acid	Turbot larvae (*Scophthalmus maximus*)	(Planas *et al.,* 2006); (Bruhn *et al.,* 2005)

If the mechanism-of-action of a probiotic bacterium is due to an antagonistic compound then development of resistance against that compound has to be taken into account. It will lead to either ineffective or even treatment failure if bacteria in the aquaculture environment including the pathogenic organisms develop resistance. Tropodithietic acid (TDA) producing bacteria have been suggested as probiotic organisms and it would therefore be relevant to study if resistance to this compound can develop. This aspect has been evaluated in this thesis and it was difficult to create resistant mutants or strains with a stable, elevated tolerance to TDA. This enhances the potential of TDA producing bacteria as probiotics in aquaculture.

2.1.2 Competition for nutrients

Competition for nutrients is considered a mechanism-of-action when using probiotic organisms in aquaculture as it can interfere with the composition of the microbiota in culture water or on surfaces on the host. The microbiota is generally dominated by heterotrophs, which compete for organic substrates as carbon and energy sources (Verschuere *et al.,* 2000b). However, only a few *in vivo* studies have been conducted within this area. Rico-Mora *et al.* (1998) isolated a bacterium due to its ability to grow in organic-poor media and showed that this bacterium protected a diatom culture against establishment of *Vibrio alginolyticus*. The authors suggested that the effect was caused by the ability of the bacterium to utilize exudates from the diatom. In another study, Verschuere *et al.* (2000a) saw that several strains had a positive effect on the survival and growth of *Artemia* juveniles and protected them from *Vibrio proteolyticus*. The effect was only observed using living

cell culture as opposed to supernatant. Hence, it was concluded that the probiotic effect was due to competition for chemicals and available energy.

Iron is essential for most organisms and although abundant, it is largely unavailable since it is bound in insoluble complexes (Hider & Kong, 2010). Hence, most microorganisms have developed strategies for acquisition of iron, and one of the most common strategies for iron acquisition is siderophore production (Hider *et al.,* 2010). Siderophores are low-molecular-weight iron chelating agents that can dissolve complex bound iron and make it available for microbial growth by up-take of the Fe-siderophore complex via specific receptors. If a probiotic bacterium uses siderophores as an inhibitory mechanism-of-action it will scavenge iron from the environment and thereby limit growth of pathogenic bacteria. Gatesoupe (1997) saw in *in vitro* experiments that *Vibrio* strain E produced siderophores, and that this strain *in vivo* protected turbot larvae (*Scophthalmus maximus* L.) from a pathogenic *Vibrio* strain P. The author proposed that that the probiotic effect was at least partly due to competition for iron. *Bacillus cereus* strain NRRL 100132 exhibited a protective effect on common carps (*Cyprinus carpio*) against *Aeromonas hydrophila* in an *in vivo* study (Lalloo *et al.,* 2007). It was later concluded through *in vitro* experiments that the positive effect was due to competition for organic carbon and iron facilitated by siderophore production, but also due to the fact that the probiotic organism had a higher growth rate as compared to the pathogenic bacteria (Lalloo *et al.,* 2010). Gram *et al.* (1999) and Spanggaard *et al.* (2001) demonstrated that several fluorescent pseudomonads were able to reduce vibriosis-induced mortality in rainbow trout (*Oncorynchus mykiss*). They suggested that siderophore production could, in part, be the cause of the disease protective effect since the fish pathogen *V. anguillarum* was not inhibited *in vitro* when the growth media were supplemented with iron. Gene expression studies supported that the probiotic effect could be due to siderophore production (Holmstrøm & Gram, 2003).

2.1.3 Competition for attachment sites

A pathogenic bacterium needs to attach and proliferate on surfaces of the host in order to cause infection. However, if these surfaces are already occupied then successful adhesion and colonization will probably be prevented. Olsson *et al.* (1992) showed that possible probiotic bacteria originating from the intestine of turbot adhered and grew better to/on fish intestinal mucus than did the pathogenic *Vibrio anguillarum*. This indicates that they perhaps would be able to compete with the pathogen for attachment sites. However, the possible probiotic organisms produced compounds that were antagonistic against the *V. anguillarum*, indicating that they perhaps could combine different mechanisms-of-actions.

Many studies have demonstrated the ability of possible probiotic organisms to adhere to intestinal mucus *in vitro*, but they did not evaluate the ability to exclude pathogenic bacteria from this surface

(Krovacek *et al.,* 1987; Olsson *et al.,* 1992; Garcia *et al.,* 1997; Jöborn *et al.,* 1997). However, Vine *et al.* (2004) found that a possible probiotic bacteria isolated from clownfish (*Amphiprion percula*) were able to both prevent attachment of *V. alginolyticus* but also remove the pathogen from the surface.

Roseobacter clade strains are among the first bacteria to colonize surfaces in marine environments (Dang & Lovell, 2000), and some have been suggested as probiotic organisms (Ruiz-Ponte *et al.,* 1999; Hjelm *et al.,* 2004a). It has been shown that *Phaeobacter* and *Ruegeria* species under specific growth conditions have a high attachment capacity and produce an antagonistic compound (TDA) (Porsby *et al.,* 2008). However, these phenotypes seen *in vitro* did not facilitate the colonization of the turbot larval gut in an *in vivo* study where *Phaeobacter* strain 27-4 were administered via live food (rotifers) (Planas *et al.,* 2006). The strain was primarily found in the culture water.

2.2 Probiotic bacteria used to control diseases in aquaculture

Vaccination has proven to be a successful approach to control diseases among many species of fish. For fish larvae, crustaceans and mollusks this strategy is not possible, and the possible effect of probiotics has therefore been the focus of several *in vivo* experiments. This section will only present a few examples of such studies. For further studies see e.g. review by Balcázar *et al.* (2006) and Kesarcodi-Watson *et al.* (2008).

2.2.1 *In vivo* probiotic studies with finfish larvae

The probiotic effect of a range of bacteria such as *Bacillus toyoi*, *Vibrio pelagius*, *Vibrio mediterranei*, *V. alginolyticus*, *V. salmonicida*, *Vibrio iliopiscarius*, *Microbacterium* sp., *Ruegeria* sp., *Pseudoalteromonas* sp., *Carnobacterium divergens*, *Streptococcus lactis* and *Lactobacillus bulgaricus*, *L. plantarum* on finfish larvae such as e.g. turbot, halibut (*Hippoglossus hippoglossus* L.) and cod (*Gadus morhua* L.) have been investigated (Gatesoupe, 1989; Gatesoupe, 1990; Garcia De La Banda *et al.,* 1992; Ringø *et al.,* 1998; Ottesen & Olafsen, 2000; Huys *et al.,* 2001; Fjellheim *et al.,* 2010). Some strains increased survival of larvae whereas others had an adverse effect leading to faster killing as compared to the control larvae only exposed to rearing water (Figure 2.2). This emphasizes the importance of testing potential probiotic bacteria in *in vivo* studies.

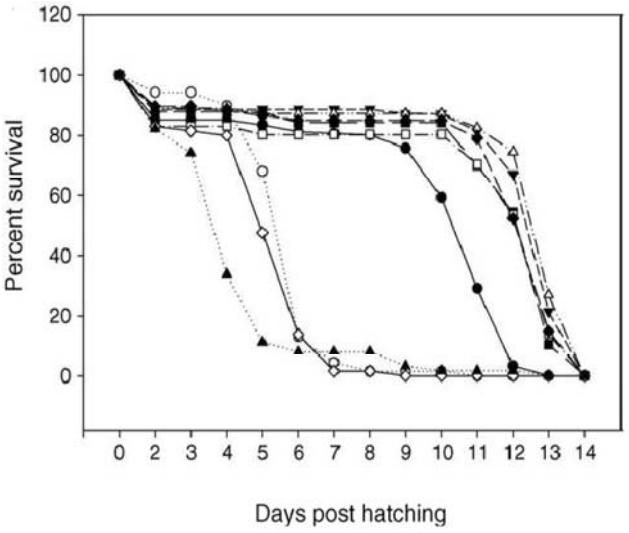

Figure 2.2 Survival of cod (*Gadus morhua* L.) larvae when they were exposed to the bacterial density 10^4 cfu/ml of seven probiotic candidates, a negative and a positive control. Negative control was *Vibrio anguillarum* O2a, positive control was non-sterilized rearing water (5.9 x 10^2 cfu/ml). Positive control (●), negative control (▲), *Vibrio anguillarum* strain RA 3-6 (○), *Ruegeria* sp. strain RA 4-1 (▼), *Pseudoalteromonas* sp. strain RA 7-14 (△), *Microbacterium* sp. strain ID 3-10 (■), *Vibrio gallicus* strain RD 5-30 (□), *Microbacterium* sp. strain ND 2-7 (◆), *Vibrio anguillarum* strain ID 4-29 (◇). Modified from (Fjellheim *et al.*, 2010)

Roseobacter strain 27-4 (reclassified as a *Phaeobacter gallaeciensis* (Martens *et al.*, 2006)) was isolated from the tank walls of a turbot rearing unit due to its antimicrobial effect against *V. anguillarum* and *Vibrio splendidus* species (Hjelm *et al.*, 2004a). The authors showed that the strain significantly increased the survival of egg yolk sac turbot larvae as compared to the non-challenged control. A similar study also showed a positive effect on survival turbot larvae due to another *Roseobacter* strain, however, the effect was not significant (Makridis *et al.*, 2005). Planas *et al.* (2006) evaluated the probiotic effect of *Phaeobacter* strain 27-4 on turbot larvae against *V. anguillarum* by incorporating the probiont and the pathogenic bacterium into live food (rotifers). When the larvae received both *Phaeobacter* strain 27-4 and *V. anguillarum* the accumulated mortality was either similar to the control (larvae fed with non-enriched rotifers) or lower than the value for larvae fed rotifers loaded with *V. anguillarum* (Figure 2.3). The authors also demonstrated that the probiont did not colonize the larval gut but it was mainly found in the culture water. This indicates that *Phaeobacter* strain 27-4 acts in the water or in surface biofilms similar to where it was isolated from.

Also mixtures of bacterial strains have a probiotic effect against finfish larvae. Makridis *et al.* (Makridis *et al.*, 2008) found that a mixture of *Shewanella* strain 2J27 and an unidentified Gram-positive bacterium increased survival of sole larvae (*Solea senegalensis*) 20 days after hatching. Cod and halibut larvae treated with a mix of *Carnobacterium divergens* V41 / *Arthrobacter* sp. / *Enterococcus* sp., and *Pseudomonas* sp. / *Cytophaga*/*Flavobacterium* sp., respectively, resulted in higher survival rates as compared to the non-treated control (Skjermo & Vadstein, 1999; Lauzon *et al.*, 2010).

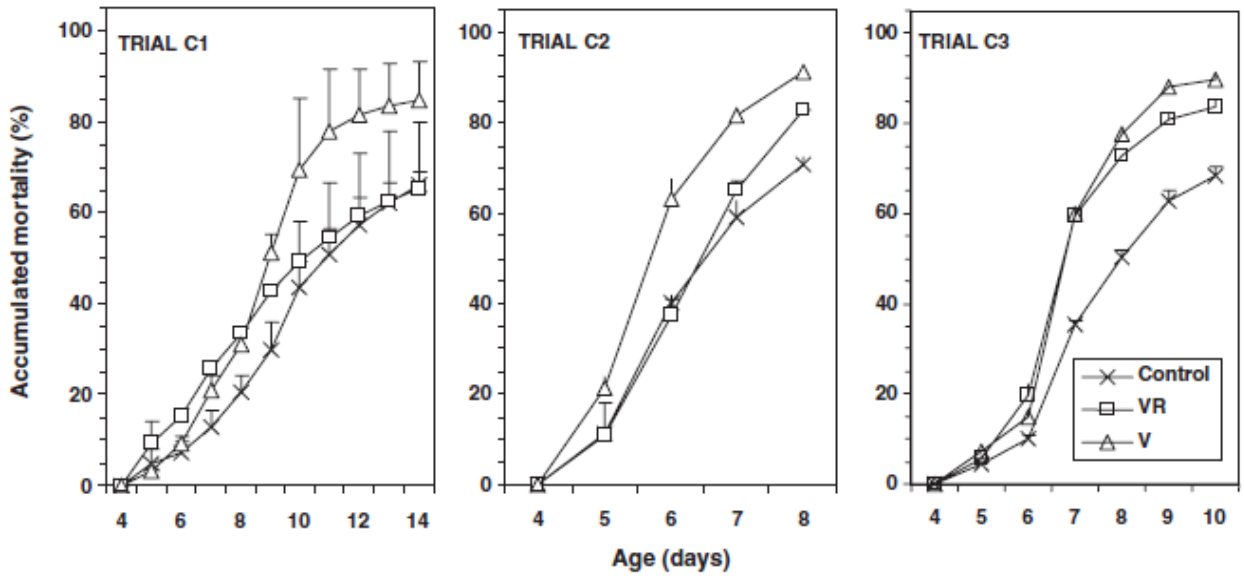

Figure 2.3 Accumulated mortalities in turbot larvae. VR: larvae were fed with rotifers loaded with *Phaeobacter* strain 27-4 (day 3, 5 and 7), with rotifers loaded with *Vibrio anguillarum* strain 90-11-287 (day 4, 6 and 8) and with non-enriched rotifers (day 9 and 10). V: larvae fed on rotifers loaded with *Vibrio anguillarum* strain 90-11-287 (day 4, 6 and 8). Control: larvae fed non-enriched rotifers. Three independent experiments are shown (Planas *et al.*, 2006)

2.2.2 *In vivo* probiotic studies with crustaceans

Most studies on crustaceans have been done on shrimps infected with a *Vibrio* species and the probiotic effect of e.g. *Bacillus*, *Vibrio* and *Pseudomonas* species (Rengpipat *et al.*, 2000; Vaseeharan *et al.*, 2003; Alavandi *et al.*, 2004; Gullian *et al.*, 2004). Infection of shrimps with *Vibrio harveyi* led to 100% mortality after 17 days, but when the shrimps were exposed to the probionts *Bacillus subtilis* strain BT23 for five days prior to infection the accumulated mortality only reached 32% (Figure 2.4) (Vaseeharan *et al.*, 2003). A short-time exposure of one hour before infection decreased the mortality to 60%. However, a synergistic effect was obtained by combining the two procedures resulting in the lowest accumulated mortality.

Commercial probiotic products for shrimps containing a single bacterial strain of or a mixture of *Bacillus* species are already available on the marked (Moriarty, 1998; McIntosh *et al.*, 2000). Castex *et al.* (2008) studied the *in vivo* probiotic effect of a commercial product containing the lactic acid bacterium *Pediococcus acidilactici* on shrimps (*Litopenaeus stylirostris*) infected with *Vibrio nigripulchritudo*. They observed a significant increase not only in the number of surviving shrimps but also in the final biomass due to probiotic treatment. *P. acidilactici* survived and withstood the conditions in shrimp digestive tract, but the beneficial bacteria did not colonize it. This might indicates that continuously addition of the probionts is necessary.

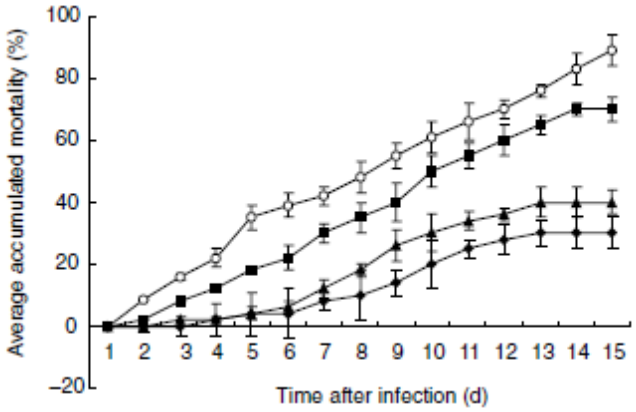

Figure 2.4 Accumulated mortalities in shrimp juveniles infected with *Vibrio harveyi* with and without probiotic treatment of *Bacillus subtilis* strain BT23. ○ control, ▲ long-term treatment, ■ short-term treatment, ♦ combined treatment (Vaseeharan *et al.*, 2003)

The *in vivo* probiotic effect of *Thalassobacter utilis* strain PM-4 on swimming crab (*Portunus trituberculatus*) larvae was repeated over a five years time period (Nogami *et al.*, 1997). The probiont, which was isolated from culture water (Nogami & Maeda, 1992), was added to the culture water at 10^5 - 10^6 cfu/ml once every 6 - 8 days. Not only did the probiont improve the survival rate of treated larvae compared to non-treated animals, also the number of individuals per m^3 increase and only one production failed as compared to 12 for the non-treated larvae (Table 2.2).

Table 2.2 Survival and production of swimming crab (*Portunus trituberculatus*) treated with *Thalassobacter utilis* strain PM-4. Modified from (Nogami *et al.*, 1997)

Year	Addition of probiont	No. of trials	Larval no. at start	Avg. survival rate (%) to 1st crab stage	Final production (individuals/m^3)	No. of production failures
1989	-	10	46,960,000	22.0	5,158	0
	+	4	20,300,000	30.4	7,703	0
1990	-	9	42,930,000	6.8	1,617	4
	+	7	30,570,000	26.7	5,838	0
1991	-	7	34,150,000	10.4	2,543	4
	+	7	34,790,000	27.9	6,938	0
1992	-	8	35,710,000	17.8	3,963	3
	+	9	37,410,000	28.8	5,994	1
1993	-	8	33,200,000	21.6	4,474	1
	+	6	26,610,000	27.7	6,150	0
Total	-	42	192,950,000	15.7	3,605	12
	+	33	149,680,000	28.2	6,397	1

2.2.3 *In vivo* probiotic studies with mollusks

Many initiatives have been tried in order to solve the problem with enormous losses of oyster larvae due to bacterial infections. Gibson *et al.* (1998) treated *Vibrio tubiashii* infected Pacific oysters

(*Crassostrea gigas*) larvae with the probiont *Aeromonas media* by adding it to the culture water. All larvae died within five days when infected with the pathogenic bacterium, but when adding the pathogen and the probiont together, the number of *V. tubiashii* decreased and resulted in complete survival of oyster larvae during the same time period. The probiotic effect is probably due to antagonistic activity of the compound 2,3-benzopyrrole (Lategan *et al.,* 2006).

Riquelme *et al.* (1997) tested 506 bacterial isolates for their *in vitro* ability to inhibit a *V. anguillarum*-related strain. A *Pseudomonas* strain, which had *in vitro* effect, and an unidentified isolate with no *in vitro* effect were tested *in vivo*. Both improved survival of Chilean scallop (*Argopecten purpuratus*) larvae from 5% to 60% when infected with a *V. anguillarum*-related strain. On the other hand, the authors showed that 2.2% of the 506 isolates were able to inhibit growth of the pathogenic bacterium *in vitro*, but *in vivo* several of them increased the mortality of the larvae. Again, this emphasizes the importance of conducting *in vivo* studies. Also Ruiz-Ponte *et al.* (1999) saw a difference between results obtained *in vitro* and *in vivo*. *Roseobacter* strain BS107 (reclassified as *Ph. gallaeciensis* (Martens *et al.,* 2006)) were able to inhibit a variety of different marine strains in *in vitro* tests. When scallop (*Pecten maximus*) were infected with *Vibrio* strain A496 and treated with *Ph. gallaeciensis* strain BS107, neither whole cells nor cell extracts protected the larvae against the infection, but the cell extract decreased the natural mortality among non-infected larvae.

2.3 Addition of probiotic bacteria

One of the challenges when it comes to use of probiotic organisms in aquaculture is addition of the probiont to the host. The following delivery routes can be used (Verschuere *et al.,* 2000b):

- Bath of the host in a suspension of the probiotic organism
- Addition of the probiotic organism to the culture water in the tanks
- Administered as addition to the artificial diet
- Addition via live feed

Few studies have been conducted where the host has been bathed in a suspension the probiont (Riquelme *et al.,* 1997; Suomalainen *et al.,* 2005), whereas most studies have administered the probiotic organism by addition to the culture water (Makridis *et al.,* 2005; Ravi *et al.,* 2007; Fjellheim *et al.,* 2010) or to the diet or feed (Planas *et al.,* 2006; Aly *et al.,* 2008). These latter two methods are probably less stressful to the fish/larvae as compared to bathing due to less handling of the animals. All these routes may require multiple, continuously additions of the probiont in order to maintain an effective level of the probiont in the rearing system. However, the idea in our research group is to make the probiotic bacteria colonize surfaces within the rearing facilities which are in contact with the culture water e.g. tank walls or biofilters and thereby creating a more stable

beneficial microbial colonization of the fish environment. This will, hopefully, restrict growth of fish pathogenic bacteria thus controlling bacterial diseases.

2.4 Conclusions from chapter 2

The main mechanisms-of-actions of probiotic organisms are probably (i) production of antagonistic compounds, (ii) competition for nutrients and (iii) competition for attachment sites, however, it is likely that the probiotic effect is due to a combination of more than one mechanism. Using probiotic bacteria in aquaculture seems like a promising way to control diseases and e.g. some members of the *Roseobacter* clade have a disease reducing effect in *in vivo* challenge trials. The *Roseobacter* clade bacteria tested in aquaculture produce the antibacterial compound TDA, and there seems to be a correlation between the production of TDA and growth of roseobacters in a biofilm stage. One could therefore hypothesize that an application in which the roseobacters is applied as biofilms could create a stable, beneficial microbiota in the fish tanks. This hypothesis is currently under investigation by PhD student Paul D'Alvise (D'Alvise *et al.,* 2010).

3 *Roseobacter* clade bacteria

Culturable bacteria of the *Roseobacter* clade was first isolated by Shiba *et al.* (1979) approximately 30 years ago, but were not classified as the newly established *Roseobacter* clade until 1991 (Shiba, 1991). Since then, new genera and species have been repeatedly added to the clade and today it contains approximately 38 genera of which many only contain one species (Brinkhoff *et al.,* 2008). The *Roseobacter* clade belongs to the α-proteobacteria and consists of both culturable and non-culturable members. Members of the clade share more than 89% identity of the 16S rRNA gene (Buchan *et al.,* 2005) and based on 16S rRNA gene similarity, they fall into different clusters (Brinkhoff *et al.,* 2008). However, at some nodes, especially those distinguishing deep branching points, the statistical power is poor, and does not provide a clear phylogenetic relationship. Therefore, Newton *et al.* (2010) compared 70 universal single-copy genes from 32 genome sequences of *Roseobacter* clade members, and this analysis resulted in five deeply branching clades (Figure 3.1). More or less the same clade-pattern was published by Tang *et al.* (2010) by comparing 1,197 single-copy genes from 17 of the same 32 genomes. The only difference was that *Maritimibacter alkaliphilus* HTCC2654 in the latter study grouped together with stains in clade 5 whereas it did not affiliate with any clusters in Figure 3.1.

Members of the *Roseobacter* clade are Gram-negative rods and some produce pigments resulting in colored colonies on agar (Shiba, 1991; Ruiz-Ponte *et al.,* 1998; Martens *et al.,* 2006; Muramatsu *et al.,* 2007; Zhang *et al.,* 2008; Vandecandelaere *et al.,* 2009):

- Pink (*Roseobacter denitrificans* and *Roseobacter litoralis*)
- Brown (*Phaeobacter gallaeciensis*, *Phaeobacter inhibens* and *Ruegeria mobilis*)
- Yellow (*Phaeobacter arcticus*)
- Blue (*Phaeobacter caeruleus*)

It has been speculated, if the pigment is produced for UV protection of the bacterial cell (a sunscreen) (Battistuzzi *et al.,* 2004). Indeed, a brown pigment such as melanin has this protective effect in other bacteria (Patel *et al.,* 1996; Hullo *et al.,* 2001; Ruan *et al.,* 2003). Alonso-Sáez *et al.* (2006) demonstrated that bacterial activity (ATP and leucine uptake) of roseobacters increased when exposed to photosynthetic radiation as compared to dark treatment. Some roseobacters do under certain conditions grow in multi-cellular star-shaped rosettes or aggregates (Figure 3.2) (Rüger & Höfle, 1992; Bruhn *et al.,* 2005) and eight out of 14 *Roseobacter* clade strains tested by Bruhn *et al.* (2007) showed this phenotype.

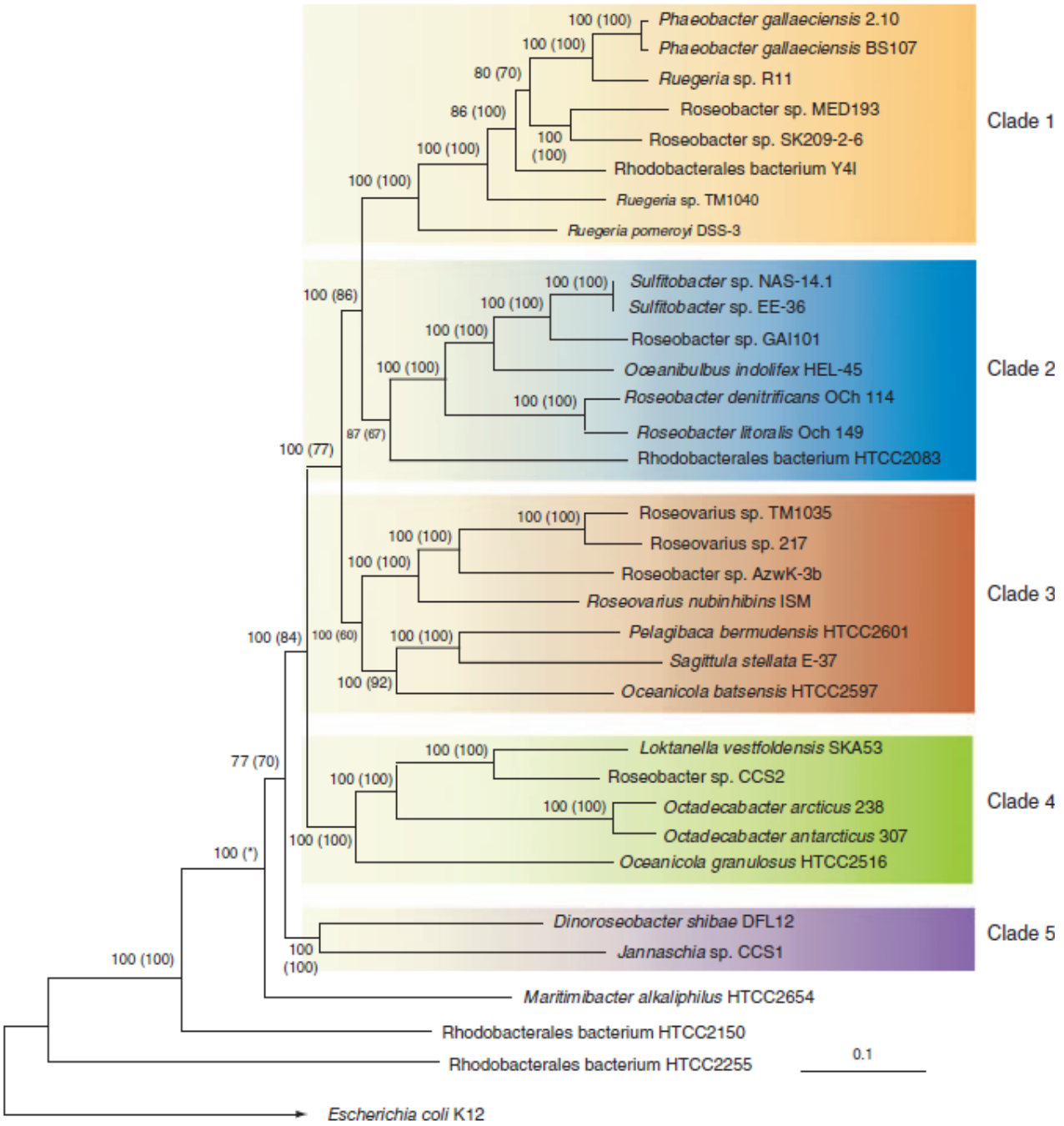

Figure 3.1 A consensus maximum likelihood tree of the 32 sequenced *Roseobacter* genomes. The alignment for tree inference was created from a concatenation of 70 universal single-copy genes contained in each of the *Roseobacter* genomes and in *Escherichia coli* strain K12, which was used as an outgroup. Bootstrap values of more than 50% for the maximum likelihood best-fit tree (200 iterations) and neighbor-joining tree (100 iterations) are listed at each node. The neighbor-joining bootstrap values are listed in parentheses. (*) demarcates nodes where the neighbor-joining tree did not agree with the maximum likelihood tree. Designated Clades 1-5 are listed to the right of the tree (Newton *et al.*, 2010)

With a few exceptions (the genus *Ketogulnicigenium* and clones from a South African goldmine), all *Roseobacter* clade members are strictly marine or hypersaline, and comparison of genome

sequences revealed that they contain genes involved in utilizing of sodium gradients and osmoregulation (Moran *et al.,* 2007). The same authors also identified 121 genes which *"make a roseobacter a roseobacter"*. Interestingly, 31% of the genes were involved in transport of compounds across the membrane including transport systems for e.g. glyoxylate (a product of algal photorespiration and organic matter photoxidation), acetate, allophanate (a breakdown product of urea in chlorophytes and bacteria), arginine, branced-chain amino-acids, ammonia, and secondary products (Moran *et al.,* 2007). Examples of transporters are porins, which covers 70% of the outer membrane of *R. denitrificans* and the authors actually describes it as a "molecular sieve" rather than a membrane (Jaroslawski *et al.,* 2009).

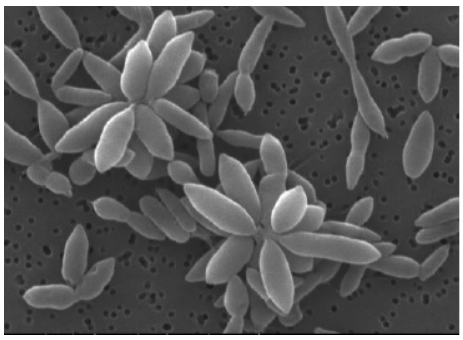

Figure 3.2 Scanning electron microscopy micrographs of *Phaeobacter* strain 27-4 grown in marine broth under stagnant conditions. Modified from (Bruhn *et al.,* 2005)

Members of the *Roseobacter* clade can utilize a broad spectrum of substrates for growth including glucose. The genomes of *Silicibacter pomeroyi* strain DSS-3, *Ruegeria* strain TM1040 and *Jannaschia* strain CCS1 all encode a complete tricarboxylic acid cycle and harbor genes for the Entner-Doudoroff pathway (Moran *et al.,* 2007). This computer-based study is in agreement with *in vivo* studies showing that *Ph. gallaeciensis* DSM 17395 and *Dinoroseobacter shibae* strain DFL12 both utilize glucose through the Entner-Doudoroff pathway and the tricarboxylic acid cycle (Furch *et al.,* 2009). Zech *et al.* (2009) also concluded that glucose is utilized in *Ph. gallaeciensis* DSM 17395 via the Entner-Doudoroff pathway by using relative abundance changes of global proteins (2-D DIGE) and metabolite (GC-MS) profiles.

Some members have alternative pathways for energy metabolism. Carbon can be derived from degradation of aromatic substrates originating from phenolic metabolites from marine plankton and liginin derivates from costal marshes (Moran & Hodson, 1994; Duval *et al.,* 1999; Faulkner, 2000). Furthermore, some strains have the ability to obtain sulfur from dimethylsulphoniopropionate (DMSP), which originates from algae, either by the cleavage pathway where the end product is the gas dimethyl sulfide that may influence the global climate, or by the demethylation/demethiolation pathway where the sulfur is incorporated into biomass (Moran *et al.,* 2003). Energy can be derived from oxidation of carbon monooxid, which is produced when sunlight oxidizes marine dissolved matter in seawater (Zafiriou *et al.,* 2003; Moran *et al.,* 2004) or by aerobic anoxygenic phototrophy where bacteria use light to get energy without generation of oxygen (Buchan *et al.,* 2005). A species

like *D. shibae* posses the ability to obtain energy both from aerobic anoxygenic phototrophy and the tricarboxylic acid cycle. A reason could be that the species can rely on the tricarboxylic acid cycle when the former is not active e.g. at night or in deeper water regions.

3.1 *Roseobacter* occurrence in marine environments

The *Roseobacter* clade is wide-spread in many different marine environments and the bacteria are exposed to different conditions such low temperature or high concentrations of salts. This indicates that the members have a great ability to adapt to a variety of life conditions.

3.1.1 Natural environments

Seawater contains approximately 10^6 cells/ml, however, it is only a small fraction of about 0.1% of all the marine bacterioplankton, which are culturable (Bernard *et al.,* 2000; Selje & Simon, 2003; Gram *et al.,* 2009). Members of the *Roseobacter* clade are a common group among non-culturable bacteria in seawater (Giovannoni *et al.,* 2000; Wietz *et al.,* 2010). Still some members are very easily culturable, and it is from these that a lot of knowledge is gained about the clade. The Global Ocean Sampling data set contains genome shotgun sequences from the ocean (Rusch *et al.,* 2007; Yooseph *et al.,* 2007). Newton *et al.* (2010) compared this data set to the genome sequences from culturable roseobacters and found that genomes from culturable roseobacters still do not provide a faithful representation of the natural roseobacter population. However, many gene systems are indeed present.

Roseobacter clade members have been found in seawater from the Pacific Ocean, Saragasso Sea, North Atlantic Ocean, Mediterranean Sea, North Sea, Antarctic Polar Front, Changjiang Estuary, Caribbean Sea and Black Sea, but also in many different marine environments like marsh and costal biofilms, various types of sediments, diatoms, corals, cephalopods, polar sea ice, sea grass, dinoflagellates and different types of algae (Buchan *et al.,* 2005; Wagner-Döbler & Biebl, 2006). An abundance of 20-30% determined by quantifying 16S rRNA gene prevalence is not uncommon in coastal waters, whereas they constitute about 15% of the bacterioplankton communities in upper mixed layer of the ocean (Buchan *et al.,* 2005). However, both higher and lower numbers have been detected. Frequencies as high as of 43% has been reported from sub-Antarctic seawater collected off Ushuaia, Argentina where the water was contaminated with the water soluble fractions of crude oil (Prabagaran *et al.,* 2007). Another study, where samples were taken at different oceanic provinces, showed that the *Roseobacter* clade constituted 1.5% to 8.2% determined by the CARD-FISH method (Wietz *et al.,* 2010). *Roseobacter* clade members are found in the upper layer of the oceans and abundance decrease with water depth. Members are have often been associated with natural algae blooms in the oceans, and here there is also a link between abundance and water

depth, but furthermore, there is a positive correlation with chlorophyll a, DMSP and total organic sulfur (González *et al.,* 2000; Alavi *et al.,* 2001). These findings suggest that the members probably prefer surfaces-attached-living as compared to free-living in the waters.

The term cosmopolitan are used in marine ecology to describe organisms that are everywhere, which is contrary to those only present in specific biogeographic niches. Some sub-groups of roseobacters appear to be associated with particular geographic areas such as temperate and arctic waters (Selje *et al.,* 2004), whereas others are abundant in all oceans (Morris *et al.,* 2002). Gram *et al.* (2010) found that culturable *R. mobilis* can be detected world-wide in almost all water masses (except Arctic and Antarctic waters). The *R. mobilis* strains are highly similar when comparing the 16S rRNA gene sequence and they cluster closely with the type strain of *R. mobilis* and strains isolated from fish farms. Furthermore, they have all retained phenotypic traits like pigmentation, ability to grow in rosettes and ability to produce the antagonistic compound tropodithietic acid (TDA). It has been discussed if all bacterioplankton are cosmopolitan, and if it is just a question of detecting them, or if the true distribution is found in the marine environments (Pedros-Alio, 2006). Some studies have found only very few cosmopolitan organisms (Pommier *et al.,* 2007), whereas others have speculated that the phenomenon is widespread and that many organisms are ubiquitous on a global scale (Finlay, 2002; Fenchel & Finlay, 2004).

3.1.2 *Roseobacter* clade bacteria in aquaculture

Aquaculture settings contain a diverse microbial community, which includes pathogenic, innocuous and beneficial bacteria, and the balance of this microbiota can be a determining factor for a successful culture environment. When considering the use of a probiotic bacterium to improve this balance, it is a good idea to use a bacterium that is already occurring in this type of environment as the bacteria will be adapted to the conditions and stresses it will be exposed to. Several studies have detected members of the *Roseobacter* clade in marine aquaculture systems either by denaturing gradient gel electrophoresis (DGGE) (Table 3.1) or by culturable isolation (Hjelm *et al.,* 2004b; Prado *et al.,* 2009). *Roseobacter* clade members have been isolated from aquaculture in this thesis work (Porsby *et al.,* 2008). The members are sometimes among the most dominating groups of bacteria found in aquaculture settings (Schulze *et al.,* 2006; Michaud *et al.,* 2009). Moreover, the type strain of the species *Ph. gallaeciensis* (strain BS107) has been isolated from the seawater from larval cultures of scallops (Ruiz-Ponte *et al.,* 1998).

Hjelm *et al.* (2004b) sampled two Spanish turbot rearing facilities once a month over a 1-year period and found *Ph. gallaeciensis* especially in spring and early summer months on the culture tank walls. Random-amplified-polymorphic-DNA (RAPD) subtyping showed that the roseobacters were very homogeneous indicating either a common, regular source or that the facilities were

colonized by this particular RAPD subtype. In this PhD work, *Ph. inhibens* and *Ph. gallaeciensis*-like strains were found to colonized the tanks in a Danish turbot rearing unit, however, these were other RAPD subtypes than the ones found in the Spanish facilities (Porsby *et al.,* 2008). Besides from *Phaeobacter* species, which were the only antagonistic genus found in the production sites, *R. mobilis* strains were isolated from the Danish turbot farm but only in the algae cultures. *Phaeobacter* strains were also isolated from an oyster hatchery in Galicia, Spain (Prado *et al.,* 2009). Despite the very different water sources used in the turbot rearing farms (the Galician Atlantic Ocean opposed to the Danish fjord Limfjorden) and the different species raised (turbot versus oysters), they all harbored an antagonistic roseobacter microbiota.

Table 3.1 Identification of roseobacters from aquaculture settings using denaturing gradient gel electrophoresis (DGGE) and culturable methodology

Aquaculture system	*Roseobacter* sp. found	Isolated from	Reference
DGGE:			
Haddock larviculture	*Roseobacter* sp.	Larvae	(Griffiths *et al.,* 2001)
Recirculation system	*Roseobacter* sp.	Biofilters	(Cytryn *et al.,* 2005)
Hatchery that culture fish and shellfish species	*Ruegeria* sp. *Roseobacter* sp. *Sulfitobacter* sp.	Abelone, clam, geoduck, blackcod, macroalgae	(Schulze *et al.,* 2006)
Cod hatchery	*Roseobacter* sp.	*Nannochloropsis* culture	(Brunvold *et al.,* 2007)
Recirculation system	*Ruegeria* sp. *Roseobacter* sp.	Biofilters, rearing water	(Michaud *et al.,* 2009)
Culturable:			
Scallop rearing facility	*Phaeobacter gallaeciensis*	Culture water	(Ruiz-Ponte *et al.,* 1998)
Turbot rearing facilities	*Phaeobacter* sp., *Ruegeria* sp.	Rotifers, tank walls, rearing water	(Hjelm *et al.,* 2004b)
Turbot rearing facility	*Phaeobacter* sp., *Ruegeria* sp.	Tank walls, rearing water, algae	(Porsby *et al.,* 2008)
Flat oyster and clams hatcheries	*Phaeobacter* sp.	Oyster larvae, culture water, tank surfaces, spat, broodstock	(Prado *et al.,* 2009)

The 16S rRNA gene is useful for phylogenetic comparison for many genera and species, however, it may not always be the best gene of choice (Dauga, 2002; Urbanczyk *et al.,* 2007). When comparing the 16S rRNA gene for Danish and Spanish roseobacters isolated from turbot rearing units, they clustered closely together (Figure 3.3a) (Porsby *et al.,* 2008). Species within the *Roseobacter* clade could in principle be difficult to separate based on 16S rRNA sequences as are other groups of organisms such as *Vibrio* species. Therefore, the gyraseB (*gyrB*) gene was in this thesis work used for phylogenetic comparison, as this gene has been used successfully to discriminate between genera and species with closely related 16S rRNA genes

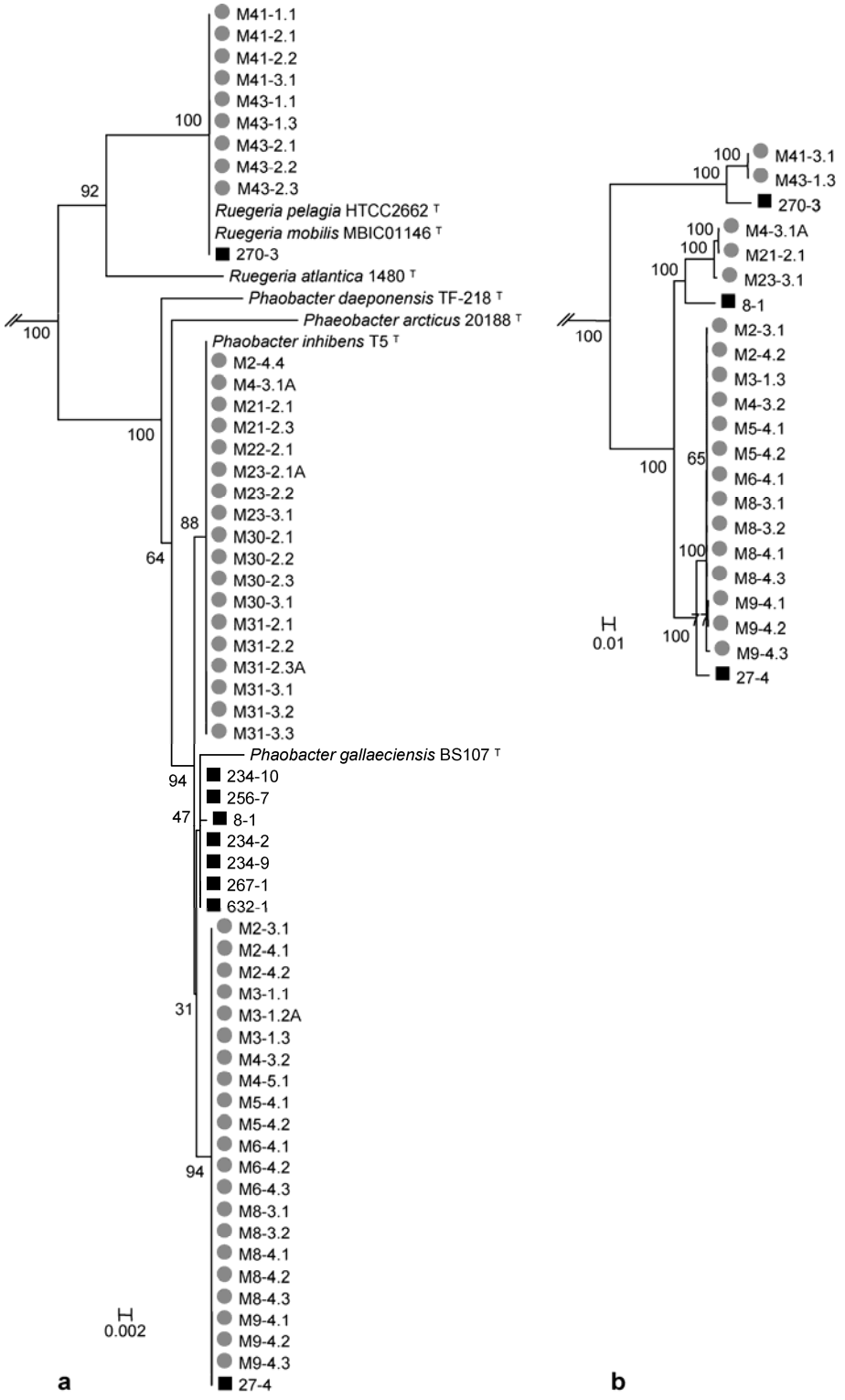

Figure 3.3 Phylogenetic tree constructed using the 16S rRNA gene (a) and gyraseB (gyrB) gene (b). Numbers at the nodes are bootstrap values from 100 replicates. Type strains *Rhodobacter capsulatus* ATCC 11166 and *Rhodobacter sphaeroides* ATCC 17023 served as outgroups in the 16S rRNA tree, and *Roseobacter denitrificans* strain Och114 and *Roseobacter litoralis* strain Och149 served as outgroups in the gyrB gene tree (not shown). ● and ■, Danish and Spanish turbot rearing farm strains, respectively. T, type strains (Porsby *et al.*, 2008)

(Fukushima *et al.,* 2002; Yáñez *et al.,* 2003; Richert *et al.,* 2005; Hannula & Hänninen, 2007). Clustering based on the *gyrB* gene resulted in the same clustering as did 16S rRNA but it gave a greater evolutionary distance between the Danish and Spanish strains (Figure 3.3b).

Surfaces in the oceans are covered with bacteria as this is a nutrient rich place for living, and therefore, there is competition for a spot on the surface. Members of the *Roseobacter* clade are among the organisms which have a preference for surfaces, and not only have they been shown to colonize a variety of inorganic and organic marine surfaces, but they are among the first bacteria to attach to a surface (Dang *et al.,* 2000; Dang & Lovell, 2002b; Dang *et al.,* 2008; Mayali *et al.,* 2008).

It is not known specifically how roseobacters attach to a surface and how this is regulated. However, it is hypothesized that possession of a holdfast structure, motility, chemotaxis, production of quorum sensing molecules and antimicrobial metabolites are some of the mechanisms which might be in use (Slightom & Buchan, 2009). Long & Azam (2001) found that antagonistic activity is more common among particle-associated bacteria as compared to free-living bacteria, indicating that the production of antimicrobials could be involved in colonization of surfaces. In general, roseobacters show great potentials for production of antagonistic compounds (Bruhn *et al.,* 2007; Martens *et al.,* 2007), but only two antibacterial compounds have been characterized from members of the clade (Table 3.2): thryptanthrin produced by *Oceanibulbus indoliflex* and TDA which is detected from *Ph. gallaeciensis*, *Ph. inhibens* and *R. mobilis*. Slightom & Buchan (2009) also mentioned a correlation between a bipyridyl pigment and antagonistic behavior of *Phaeobacter* sp. strain Y4I. However, it is not clear if the antagonism is caused by the pigment or by another compound.

Both *Phaeobacter* strain 27-4 and *Ph. gallaeciensis* strain SK2.10 colonize surfaces in *in vitro* models (Rao *et al.,* 2005; Bruhn *et al.,* 2006; Rao *et al.,* 2006). Rao *et al.* (2005; 2006) showed that *Ph. gallaeciensis* strain SK2.10 effectively outcompete other marine bacteria including the strong biofilm-former, *Pseudoalteromonas tunicata*, on marine algae. Production of TDA is assumed to be the primary mechanism as TDA has been shown to cause an antagonistic effect of both *Ph. gallaeciensis* strain SK2.10 and *Phaeobacter* strain 27-4 (Bruhn *et al.,* 2005; Gram *et al.,* 2010). A great variety of bacteria originating from marine environments and bacteria known to cause diseases in fish and humans were all inhibited by crude cell cultures, washed cells, or supernatants from *Ph. gallaeciensis*, *Ph. inhibens*, *R. mobilis* or different *Phaeobacter* and *Ruegeria* strains in diffusion assays (Table 3.3). In this PhD study, strains randomly isolated from a turbot rearing facility were all inhibited by *Phaeobacter* and *Ruegeria* strains originating from the same facility (Porsby *et al.,* 2008). Therefore, one should be aware that if such bacteria are deliberately added as

probiotic organism, they might not just inhibit the pathogenic agents, but could perhaps also alter the general microbiota.

Table 3.2 Antagonistic compounds produced by *Roseobacter* clade members

***Roseobacter* clade member**	**Strain**	**Origin**	**Antagonistic compound**	**Reference**
Oceanibulbus indoliflex	HEL-45	North Sea	Thryptanthrin	(Wagner-Döbler *et al.,* 2004)
Phaeobacter gallaeciensis	BS107	Scallop larvae culture	Tropodithietic acid	(Ruiz-Ponte *et al.,* 1998)
Phaeobacter inhibens	T5, M23-3.1 etc.	German Wadden Sea, Turbot rearing unit	Tropodithietic acid	(Brinkhoff *et al.,* 2004); (Porsby *et al.,* 2008)
Phaeobacter sp.	27-4, M2-3.1etc.	Turbot rearing units	Tropodithietic acid	(Hjelm *et al.,* 2004a); (Porsby *et al.,* 2008)
Phaeobacter sp.	Y4I	Unknown	Unknown	(Slightom *et al.,* 2009)
Ruegeria mobilis	NBRC101030 M43-2.3	Marine biofilms, Turbot rearing unit	Tropodithietic acid	(Porsby *et al.,* 2008); (Gram *et al.,* 2010)
Ruegeria sp.	TM1040	Dinoflagellate	Tropodithietic acid	(Geng *et al.,* 2008)

D'Alvise *et al.* (2010) showed that the fish pathogenic bacteria *Vibrio anguillarum* strain 90-11-287 was killed rapidly by *Phaeobacter* strain M23-3.1 (Figure 3.4). The effect was observed both when the *Phaeobacter* stain was applied attached to a peg and as suspended cells. Consistent with this, *Phaeobacter* strain 27-4 showed a killing effect and a mutant devoid of TDA production did not kill *V. anguillarum* strain 90-11-287, indicating that TDA caused the antagonistic effect. This study shows that the probiont might be added to an aquaculture system as attached bacteria on solid surfaces.

Table 3.3 Inhibition of various strains by *Phaeobacter* spp. and *Ruegeria* spp. determined by well diffusion assays using crude cell cultures, washed cells, or supernatants

Target strain	Inhibited by	Reference	Target strain	Inhibited by	Reference
Marine bacteria			Fish pathogens		4
Erythromicrobium ramosum	T5, BS107	1	*Vibrio anguillarum*	M23-3.1, M43-2.3, PP-154, TM1040	4, 5, 6
Leisingeria methylohalidivorans	T5, BS107	1	*Vibrio vulnificus*	M23-3.1, M43-2.3, PP-154	4, 5
Colwellia maris	T5, BS107	1	*Vibrio splendidus*	M23-3.1, M43-2.3, PP-154	4, 5
Psychroserpens burtonenis	T5, BS107	1	*Vibrio harveyi*	M23-3.1, M43-2.3, PP-154	4, 5
Tenacibaculum	T5, BS107	1	*Vibrio aestuarianus*	PP-154	5
Zobellia uliginosa	T5, BS107	1	*Vibrio alginolyticus*	PP-154	5
Aeromicrobium fastidiosum	T5, BS107	1	*Vibrio fischeri*	PP-154	5
Pseudonocardia alni	T5, BS107	1	*Vibrio fluvialis*	PP-154.	5
Alteromonas marina	SCH0407	2	*Vibrio logei*	PP-154	5
Shewanella oneidensis	SCH0407	2	*Vibrio mimicus*	PP-154	5
Bacillus atrophaeus	SCH0407	2	*Vibrio parahaemolyticus*	PP-154	5
Aquaculture isolates			*Vibrio pelagiusproteolyticus*	PP-154	5
Halomonas sp./*Cobetia marina*	*Phaeobacter* spp. *Ruegeria* spp.	3	*Vibrio natriegens*	PP-154	5
Micrococcus sp.	*Phaeobacter* spp. *Ruegeria* spp.	3	*Vibrio neptunius*	PP-154	5
Pseudomonas sp.	*Phaeobacter* spp. *Ruegeria* spp.	3	*Vibrio tapetis*	PP-154	5
Pseudoalteromonas sp.	*Phaeobacter* spp. *Ruegeria* spp.	3	*Vibrio tubiashii*	PP-154	5
Marinomonas sp.	*Phaeobacter* spp. *Ruegeria* spp.	3	*Aeromonas salmonicida*	M23-3.1, M43-2.3, PP-154	4, 5
Rhodococcus sp.	*Phaeobacter* spp.	3	*Aeromonas hydrophila*		
Olleya marilimosa	*Phaeobacter* spp. *Ruegeria* spp.	3	*Tenacibaculum martimum*	M23-3.1, M43-2.3, PP-154	4, 5
Kordia algicida	*Phaeobacter* spp. *Ruegeria* spp.	3	*Edwardsiella tarda*	PP-154	5
Human pathogens		5	*Pseudomonas anguilliseptica*	PP-154	5
Bacillus subtilis	T5	1	*Lactococcus piscium*	M23-3.1, M43-2.3	4
Staphylococcus aureus	T5	1			
Escherichia coli	T5	1			

1: (Brinkhoff *et al.*, 2004); 2: (Bhattarai *et al.*, 2006); 3: (Porsby *et al.*, 2008); 4: (D'Alvise *et al.*, 2010); 5: (Prado *et al.*, 2009); 6: (Bruhn *et al.*, 2007)

T5: *Phaeobacter inhibens*; BS107 and SCH0407: *Phaeobacter gallaeciensis*; M23-3.1 and PP-154: *Phaeobacter* sp.; M43-2.3: *Ruegeria mobilis*; TM1040: *Ruegeria* sp.

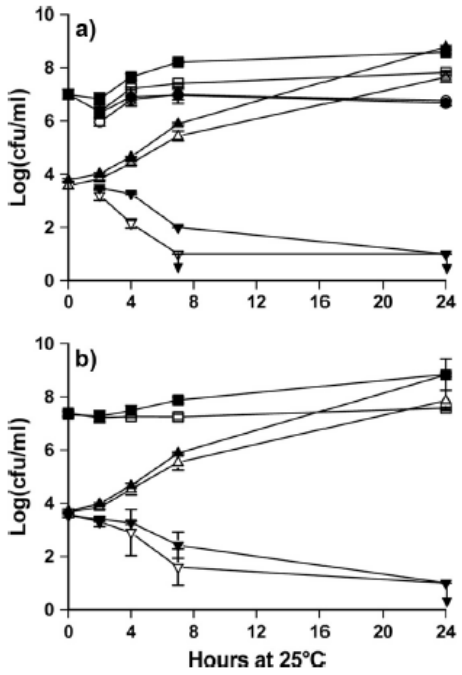

Figure 3.4 *Vibrio anguillarum* strain 90-11-287 was exposed to *Phaeobacter* sp. strain M23-3.1 that was introduced as attached cells on pegs (a), or as suspended cells (b). Counts of *V. anguillarum* exposed to *Phaeobacter* in marine broth (MB) (▼) and in Instant Ocean supplemented with glucose and amino acids (IO+) (▽); negative controls were unexposed *V. anguillarum* in MB (▲) and in IO+ (△). Growth of *Phaeobacter* was measured in the culture liquid (MB ■, IO+ □) and on the pegs (MB ●, IO+ ○). Points are averages of four determinations (duplicates in two independent trials) and error bars are standard deviation of the mean (D'Alvise *et al.*, 2010)

3.2 Conclusions from chapter 3

Roseobacter clade members are strictly marine or hypersaline and they can be found in a variety of different marine environments both as culturable isolates and as non-culturable clones. The members are often associated different types of surfaces or particles which could indicate a strong preference for this lifestyle. The fact that some roseobacters are naturally occurring in many aquaculture settings, that they are easily culturable, and that *Phaeobacter* and *Ruegeria* strains have an antagonistic effect against a range of other bacteria make them promising probiotic candidates. The antagonistic effect of *Phaeobacter* and *Ruegeria* species is primarily due to production of TDA.

4 Production of tropodithietic acid by *Roseobacter* clade strains

Some *Roseobacter* clade members have an antibacterial effect, which is believed to be due to the secondary metabolite tropodithietic acid (TDA). It has been hypothesized that TDA causes the "disease reducing effect" when the probiont *Phaeobacter* strain 27-4 is added to turbot larvae infected with *Vibrio* (D'Alvise *et al.,* 2010). Therefore, it is important from an applied point of view to elucidate both the nature of the antagonistic compound that causes the effect, and to understand how environmental and genetic factors influence TDA production.

4.1 Tropodithietic acid

Tropolones is a family of non-benzenoid aromatic compounds containing a seven-membered ring with a hydroxyl and a carbonyl group. Some derivates of this family have antagonistic effect against bacteria, viruses and fungi (Trust, 1975; Kintaka *et al.,* 1984; Morita *et al.,* 2002; Budihas *et al.,* 2005). TDA (Figure 4.1a) is such a derivate, and contains a disulfide bridge. It is likely to exist in a tautomeric form together with thiotropocin (Figure 4.1b) (Greer *et al.,* 2008). TDA has a pK_a value of 2 and is water soluble at neutral pH (7). The antibacterial activity of supernatant containing TDA from *Phaeobacter* strain 27-4 declined with increasing temperature. For example, no activity of the supernatant was detected after storage at 37°C for two days, whereas activity was stable at 5°C for up to eight days and for 3 months at -80ºC (Bruhn *et al.,* 2005).

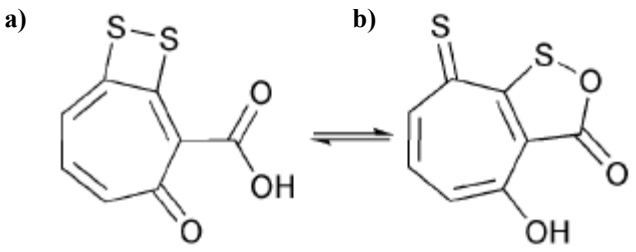

Figure 4.1 Structure of the antibacterial compounds tropodithietic acid (a) and thiotropocin (b). Modified from (Thiel *et al.*, 2010)

Production of TDA is not a common feature of all *Roseobacter* clade members as the compound has only been detected from the species *Phaeobacter gallaeciensis*, *Phaeobacter inhibens* and *Ruegeria mobilis*. *Silicibacter* strain TM1040 also produces TDA, however, it is currently being discussed if the *Silicibacter* genus should be reclassified to the *Ruegeria* genus (the strain will in this thesis be named *Ruegeria* strain TM1040) (Brinkhoff *et al.,* 2008; Newton *et al.,* 2010). Other roseobacters such as *Roseobacter denitrificans*, *Roseobacter litoralis*, *Roseovarius tolerans*, *Leisingera methylohalidivorans*, *Marinovum Agricola*, *Silicibacter pomeroyi* and *Sulfitobacter pontiacus* do not produce this antibacterial compound (Gram *et al.,* 2010; Geng & Belas, 2010). This is in agreement with Newton *et al.* 2010(#261) who only found genes for TDA biosynthesis in genomes

of *Ruegeria* strain TM1040, *Ph. gallaeciensis* strains SK2.10 and BS107, but not in 29 other genomes of *Roseobacter* clade members.

TDA producing strains are also found outside the *Roseobacter* clade. A *Pseudovibrio* strain JE062 isolated from a marine sponge also produce TDA, however, it belongs to the same order (*Rhodobacterales*) as roseobacters do (Geng *et al.,* 2010). Thiotropocin (or TDA) is produced by a soil bacteria identified as *Pseudomonas* (strain CB-104) (Tsubotani *et al.,* 1984; Kintaka *et al.,* 1984) and *Caulobacter* strain PK654 isolated from marine microalgae (Kawano *et al.,* 1997; Kawano *et al.,* 1998). It is likely that *Caulobacter* strain PK654 belongs to the genera *Phaeobacter* or *Ruegeria* as biochemical tests indicate this (Gram negative, nonfermentativ / nonoxidative, motile rods with positive catalase and oxidase reactions and they form brown colonies on marine agar). In addition, the GC content of the *Caulobacter* genus is normally 62-67 mol% (Holt *et al.,* 1994) but strain PK654 contains only 58 mol%, which is similar to the GC content of *Ph. gallaeciensis* (57.6-58 mol%) (Ruiz-Ponte *et al.,* 1998). *R. mobilis* has a slightly higher GC content (58.5 mol%) (Muramatsu *et al.,* 2007). Hence, strain PK654 may not be a new species within the genus *Caulobacter* as proposed by Kawano *et al.* (Kawano *et al.,* 1997), but a *Ph. gallaeciensis* strain.

4.2 Conditions facilitating production of tropodithietic acid

Roseobacter clade strains do not produce TDA under all laboratory conditions but require specific environmental settings. A physical parameter such a shaking (aeration) is important for TDA production. For *Phaeobacter* strain 27-4 grown at stagnant conditions there is a positive correlation between production of brown pigment, formation of star-shaped aggregates (rosettes), ability to inhibit *Vibrio anguillarum* strain 90-11-287 and production of TDA (Figure 4.2 c and d) (Bruhn *et al.,* 2005). However, none of these phenotypes appear if the strain is grown with shaking. Similar correlations have to the best of my knowledge not been reported for other tropolone compounds. However, production of pigment and antibacterial activity are only observed at stagnant conditions for *Bacillus licheniformis* strain EI-34-6 and not when grown with shaking (Yan *et al.,* 2002; Yan *et al.,* 2003). Yang *et al.* (2007) noticed a connection between growth in aggregates and production of the pigment violacein, which have antibacterial activity, as production was most pronounced at stagnant conditions.

Separation of supernatant from *Phaeobacter* strain 27-4 into different fractions showed that the fraction containing TDA was antibacterial, whereas the brown pigment fraction had no activity. Therefore, the brown pigment and TDA are two different compounds but the pigment appears to be an indicator for presence of TDA when the culture is grown in marine broth. It is unknown what the brown pigment is but it could be a melanin compound as production of this pigment is known from

other bacteria (Ruan *et al.,* 2003; Dastager *et al.,* 2006; Huang *et al.,* 2009). It can also be speculated if polymerization of TDA leads to the brown pigment as these two phenotypes co-occur (Bruhn *et al.,* 2007).

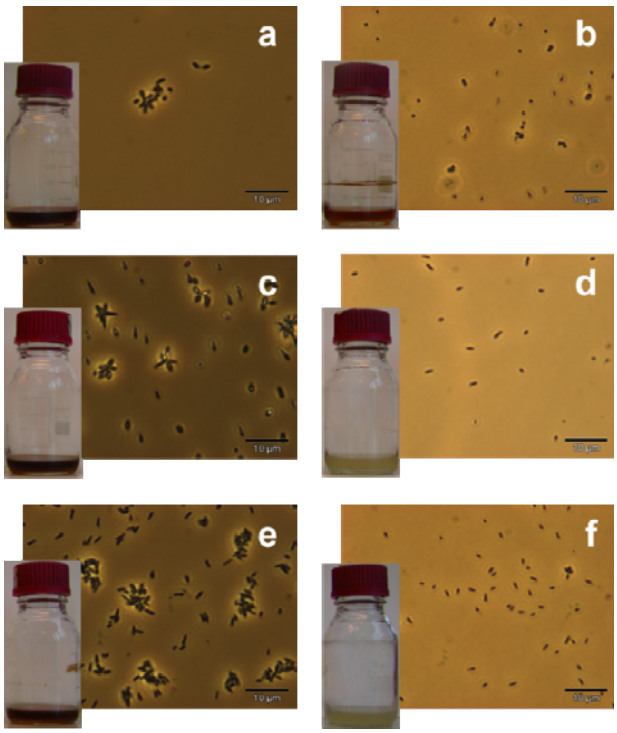

Figure 4.2 Cell morphology of and pigment formation by *Roseobacter* clade strains grown in marine broth under static (0 rpm) (a, c and e) or shaking (200 rpm) (b, d and f) conditions. The Danish *Phaeobacter* sp. strain M23-3.1 (a and b), the Spanish *Phaeobacter* sp. strain 27-4 (c and d), and the Danish *Ruegeria* sp. strain M43-2.3 (e and f) are shown. Microscopy pictures are from phase-contrast microscopy at 1000x magnification. Bars, 10 µm (Porsby *et al.,* 2008)

Not all TDA producing roseobacters have this demanding physical requirement for production of TDA. The use of TDA producing roseobacters as fish larvae probiotics is the long term aim, and stringent stagnant conditions are not likely to be found in a fish tank. Work from this PhD study showed that all *Phaeobacter* strains isolated from Danish or Spanish turbot rearing facilities (except strain 27-4) display the mentioned phenotypes simultaneously both during shaking and stagnant conditions (Figure 4.2 a and b and Figure 4.3) (Porsby *et al.,* 2008). However, the size of inhibition zones against *V. anguillarum* strain 90-11-287 in well diffusion assay and the amount of TDA produced are greatest at stagnant growth conditions. In contrast, all *R. mobilis* strains tested only produce brown pigment, grow in rosettes, inhibit *V. anguillarum* strain 90-11-287 and produce TDA when grown under stagnant conditions in marine broth (Figure 4.2 e and f and Figure 4.3). So interestingly, *Phaeobacter* strain 27-4 shows highest similarity to other *Phaeobacter* strains when comparing their 16S rRNA genes, but phenotypically it resembles *R. mobilis* strains. *Ph. inhibens* strain T5 also produce TDA under both growth conditions (Gram *et al.,* 2010).

The brown pigment and probably also TDA are mainly produced in the surface-media interface of the stagnant cultures of *Phaeobacter* strain 27-4 and *R. mobilis*. It can be hypothesized that requirements for specific atmosphere conditions e.g. oxygen concentrations causes this phenomenon, or that the strains have a preference for the surface tension found in this air-liquid

interface. However, either these hypotheses are not correct, or different species vary with regard to which conditions facilitate TDA production, as *Ph. gallaeciensis* and *Ph. inhibens* display these phenotypes both when grown at stagnant and shaken conditions. As this positive correlation between production of TDA, ability to grow in rosettes or aggregates and ability to attach to a surface exists (Figure 4.2 and Figure 4.3) (Bruhn *et al.,* 2007), it can indicate that biofilm formation is an important issue. To investigate this correlation, it can be studied if TDA negative mutants also lost the ability to attach to a surface. It can also be speculated if TDA producing bacteria encode genes for surface proteins unique to this group of bacteria. This could perhaps be studied by comparing genomes of TDA producing bacteria (e.g. *Ph. gallaeciensis* strain BS107 and *Ruegeria* strain TM1040) to *Sulfitobacter* sp. strain EE-36 which at stagnant growth conditions form rosettes and attach very well to a surface (Bruhn *et al.,* 2007) but do not produce TDA (Geng *et al.,* 2010).

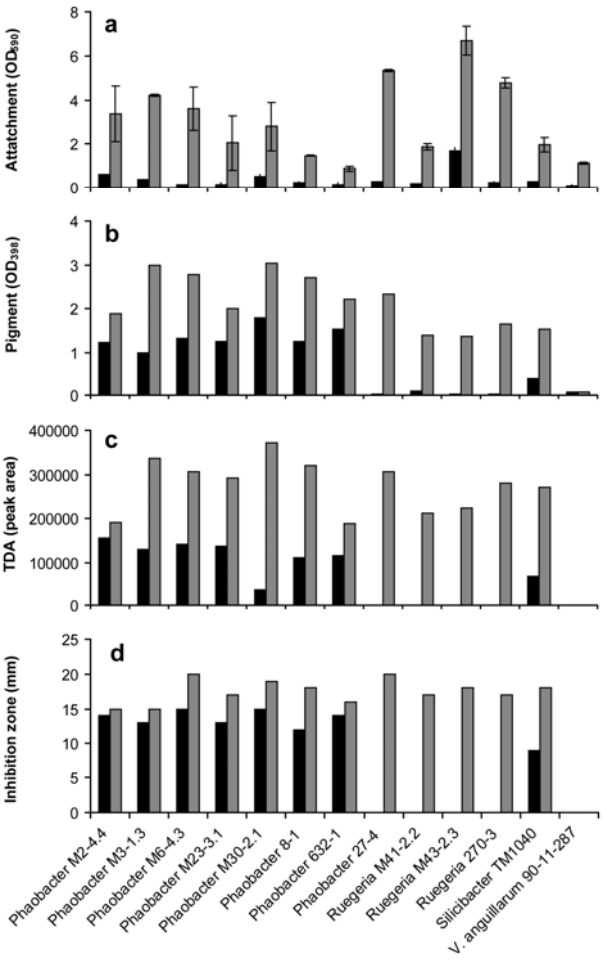

Figure 4.3 Attachment (a), production of pigment (b), tropodithietic acid (c), and ability to inhibit *Vibrio anguillarum* strain 90-11-287 in well diffusion assay (d) of *Roseobacter* clade strains and *V. anguillarum* strain 90-11-287 grown under shaking (200 rpm) (black bars) or stagnant (0 rpm) (gray bars) conditions. The attachment assay was conducted in duplicates, and error bars represent standard deviations. Strains are grown in marine broth for three days. In the well diffusion assay, the diameter of the well itself has been subtracted from the diameter of the inhibition zone (Porsby *et al.,* 2008)

Temperature and marine salts also influence production of TDA in *Phaeobacter* strain 27-4 when grown in marine broth (Bruhn *et al.,* 2005). Cultures incubated at 15 to 30°C produced the antibacterial compound, whereas 5, 10 and 37°C did not facilitate the production. Only minor

bacterial activity was detected from cultures grown in marine broth where insoluble compounds (salts) are removed.

4.3 Biosynthesis of tropodithietic acid

Secondary metabolites are all produced from a relatively small number of key intermediates. These arise from the primary metabolic pathways that are shared by all bacteria, namely the pathways for production or metabolism of primary metabolites i.e. carbohydrates, proteins, and nucleic acids. Example of such a key intermediate is shikimic acid, which can be used in the production of aromatic amino acids and other aromatic compounds (Mann, 1994). Both TDA and thiotropocin are aromatic compounds (Figure 4.1). Cane *et al.* (1992) showed that labeled glucose is converted into phenylacetic acid through shikimate by the shikimate-chorismate pathway, and the authors suggest two plausible routes to thiotropocin where phenylacetic acid is altered by oxidative ring expansion followed by further oxygen-sulfur exchange (Figure 4.4). The same pathways are identified by Thiel *et al.* (2010), however, they propose that a bound phenylacetyl-CoA substrate is more likely than a free phenylacetic acid.

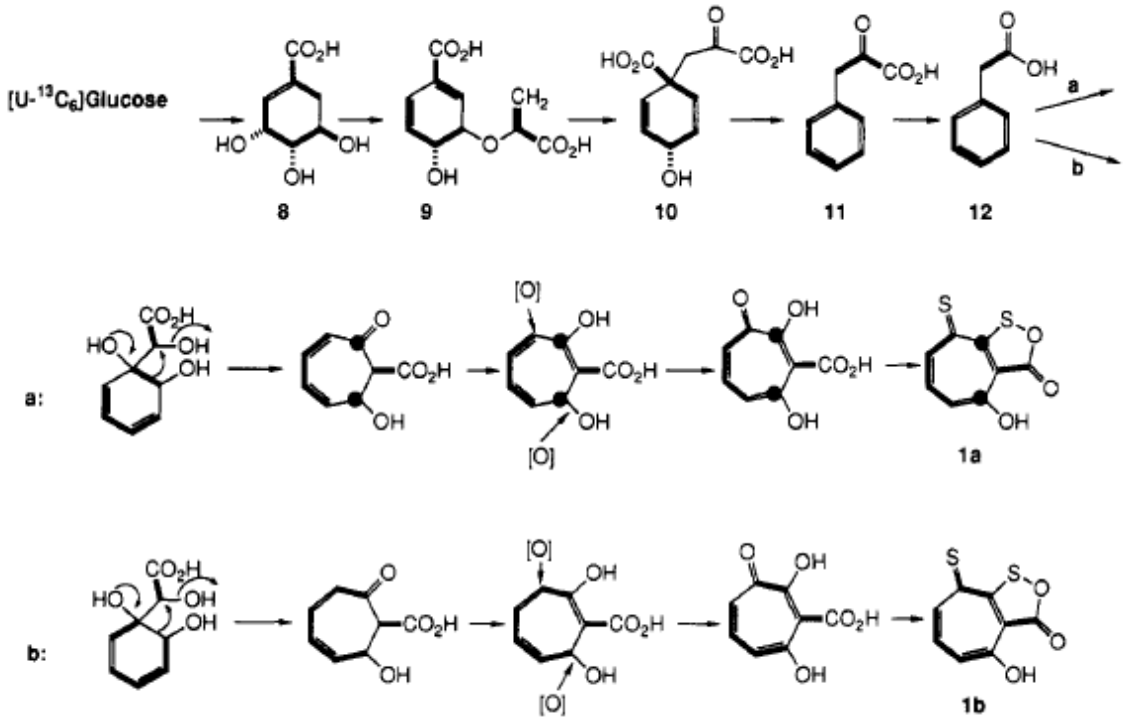

Figure 4.4 Proposed biosynthesis of thiotropocin. Shikimate (8) is converted into phenylacetic acid (12) via the shikimate-chorismate pathway, and phenylacetic acid (12) then undergoes oxidative ring expansion to thiotropocin (1a and 1b) (Cane *et al.*, 1992)

No studies have shown how the two sulfur atoms are added onto the TDA molecule, however, it has been speculated if dimethylsulphoniopropionate (DMSP) produced by algae in the oceans is the

source in natural environments (Bruhn *et al.,* 2007; Geng *et al.,* 2008; Geng *et al.,* 2010). This is a possible source as TDA producing roseobacters contain genes involved in the utilization of DMSP (*dmdA* and *dddP*) (Newton *et al.,* 2010). However, conflicting results are published as Geng *et al.* (2008) did not observe TDA production from *Ruegeria* strain TM1040 after addition of DMSP, whereas Geng & Belas (2010) found a two-fold increase by addition of DMSP. However, this does not explain why roseobacters produce TDA in laboratory media such marine broth (Bruhn *et al.,* 2005; Geng *et al.,* 2008; Porsby *et al.,* 2008) which does not contain DMSP. Hence, other sulfur sources can be used in the biosynthesis. Marine broth is rich in amino acids and sulfur, and Geng *et al.* (2008) demonstrated that addition of the amino acid cysteine as the only sulfur source induced TDA production.

4.3.1 Genes involved in biosynthesis of tropodithietic acid

Genes involved in TDA biosynthesis has been identified by random transposon insertion mutagenesis in *Phaeobacter* strain 27-4 and *Ruegeria* strain TM1040 (Table 4.1) (Geng *et al.,* 2008). The main analysis were carried out on *Ruegeria* strain TM1040 as this strain is genome sequenced, and this is probably why e.g. all the *tdaA* to *tdaF* genes (see later this section) is not identified in *Phaeobacter* strain 27-4.

The only regulatory gene found among mutants lacking TDA production was *tdaA*, which has homology to LysR regulators (Geng *et al.,* 2008). LysR regulatory proteins are global transcriptional regulators acting as either activators or repressors of single or operonic genes (Maddocks & Oyston, 2008). They regulate genes involved in e.g. cell division (Lu *et al.,* 2007), motility (Lehnen *et al.,* 2002), quorum sensing (Sperandio *et al.,* 2002), virulence factors (Sheehan & Dorman, 1998) and sulfur utilization (Jovanovic *et al.,* 2003). This indicates that biosynthesis of TDA at a transcriptional level is regulated by a LysR regulator homolog.

The gene *tdaA* is a part of a gene organization, which includes the genes *tdaA* to *tdaF*, and it is present in both *Ruegeria* strain TM1040, *Ph. gallaeciensis* strain BS107 and SK2.10 and *Pseudovibrio* strain JE062 (Figure 4.5). In *Ruegeria* strain TM1040 these genes are localized on a plasmid. Searching the genomes of *Ph. gallaeciensis* strains the same organization of *tdaA* to *tdaF* were found, however, they were localized on the chromosome. Also, a partly assembled genome of *R. mobilis* strain F1926, which was isolated on a global research expedition (Gram *et al.,* 2009; Gram *et al.,* 2010), harbors this organization of *tdaA* to *tdaF* (unpublished data). From an applied point of view, it is an advantage that the genes involved in TDA production are located on the chromosome since loss of TDA production by spontaneous loss of the plasmid (Geng *et al.,* 2008) will not occur. Spontaneous non-pigmented mutants which no longer had antibiotic activity were

also observed for *Ph. inhibens* strain T5 (Brinkhoff *et al.,* 2004), indicating that perhaps genes involved in TDA production are localized on a plasmid in this strain.

Table 4.1 Genes identified in biosynthesis of tropodithietic acid in *Ruegeria* strain TM1040 and *Phaeobacter* strain 27-4. Modified from (Geng *et al.,* 2008)

Function	***Ruegeria* strain TM1040**		***Phaeobacter* strain 27-4**	
	Gene	**Function**	**Gene**	**Function**
Ring precursor, oxidation, and expansion	*paaK*	Phenylacetate oxidoreductase		
	paaI	Phenylacetate oxygenase		
	paaJ	Phenylacetate oxygenase		
	tdaD	4-Hydroxybenzoyl-CoA thioesterase		
	tdaE	Acyl-CoA dehydrogenase		
	tdaB	Beta-etherase	*tdaB*	Beta-etherase
	tdaC	Prephenate dehydratase		
Sulfur metabolism and addition	*malY*	C-S lyase (cystathionase); Amino transferase		
	tdaH	Sulfite oxidase domain protein		
	cysI	Sulfite reductase		
			metF	5-Methyltetrahydrofolate–Homocysteine; *S-m*ethyltransferase
CoA metabolism	*tdaF*	Phosphopantothenoylcysteine decarboxylase		
			tdbA	D-β-Hydroxybutyrate dehydrogenase
			tdbB	Phosphate acetyltransferase
			tdbC	Lytic transglycosylase, peptidase C14
			traI	TraI, type IV (Vir-like) secretion
			tdbD	Type I secretion target repeat protein
			tdbE	Oligopeptide/dipeptide ABC transporter
Regulatory mechanism	*tdaA*	LysR substrate-binding domain protein	*tdaA*	LysR substrate-binding domain protein

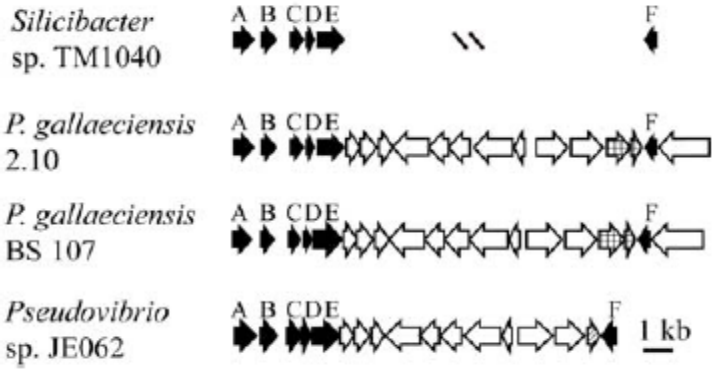

Figure 4.5 Arrangement of the genes *tdaA* to *tdaF* involved in production of tropodithietic acid in *Ruegeria* strain TM1040, *Phaeobacter gallaeciensis* strain BS107 and SK2.10 and *Pseudovibrio* strain JE062. ⋀⋀ indicates a break in the region. Modified from (Geng *et al.*, 2010)

Due to the arrangement of the *tdaA* to *tdaF* genes in *Ruegeria* strain TM1040, one could hypothesize that *tdaA* regulates *tdaB* to *tdaF*. Geng & Belas (2010) indicates that *tdaA* and *tdaB* are arranged in an operon and that *tdaCDE* constitute a separate operon. The gene organization indicates that the *tdaF* gene forms a putative operon with the gene next to it, as there are only 35 nucleotides between them. This gene is a metabolism gene encoding an aldehyde dehydrogenase. Under stagnant growth conditions, which facilitates TDA production in *Ruegeria* strain TM1040, *tdaA* is expressed constitutively whereas the amount of *tdaCDE* and *tdaF* RNA increase significantly as compared to conditions where TDA is not being produced (shaking) (Geng *et al.*, 2010). Also, in nine of 11 TDA negative mutants the ability to transcribe *tdaC* is restored by placing TDA producing strains in their proximity or by addition of exogenous TDA. The authors suggest that TDA (or a late-stage chemical intermediate in TDA biosynthesis) induce *tda* gene expression and that the compound functions as an autoinducer of its own synthesis (Geng *et al.*, 2010).

Attempts were made in this PhD study to use Nothern blot to reveal the gene organization of *tdaA* to *tdaF* in *Ph. gallaeciensis* strain BS107 and *Ruegeria* strain F1926, and to quantify gene expression of *tdaB* in the same two strains using quantitative-Real-Time-PCR. However, the methods did not work – probably due to low expression levels of the genes. Another obstacle was to obtain good quality and quantity RNA from those two bacteria, and several RNA purification kits and growth media were tried out.

4.4 Conclusions from chapter 4

The antibacterial compound TDA is only produced by *Ph. gallaeciensis*, *Ph. inhibens*, *R. mobilis*, thus, this is not a phenotype defining all *Roseobacter* clade members. *Ph. gallaeciensis* and *Ph. inhibens* strains produce TDA both when grow at stagnant and shaking conditions, whereas *R. mobilis* and *Phaeobacter* strain 27-4 only produce the antagonistic compound at shaking conditions. This versatility of *Phaeobacter* strains (except strain 27-4) makes them interesting in an applied perspective as only a few sites in a fish tank will be stagnant, and it is believed that this group will have probiotic effect in aquaculture settings due to TDA production. Therefore, it is important to gain knowledge about e.g. which bacteria are sensitive to TDA and what the mechanism-of-action of TDA against other bacteria is (chapter 5). The biosynthesis pathway of TDA is not fully known, but the compound is probably biosynthesized from phenylacetyl-CoA by oxidative ring expansion and oxygen-sulfur exchange. However, the exact way the sulfur atoms is added to the molecule is not identified. The sulfur source for TDA production includes e.g. cysteine (at least for *Ruegeria* strain TM1040) whereas the algal product DMSP is a more questionable source under laboratory conditions.

5 Tropodithietic acid as an antimicrobial compound

It is believed that the fish probiotic effect caused by *Roseobacter* clade strains is due to production of the antagonistic compound tropodithietic acid (TDA) (Bruhn *et al.*, 2005; D'Alvise *et al.*, 2010). To use TDA producing strains as probionts it is important to understand the response of the target bacteria when exposed to TDA or the probiotic bacteria. So far, resistance to novel antibiotics has developed within a couple of years after introduction (Figure 5.1) (Clatworthy *et al.*, 2007). It is therefore a key issue in the use of TDA producing probiotic bacteria, to address if target bacteria develop resistance to TDA.

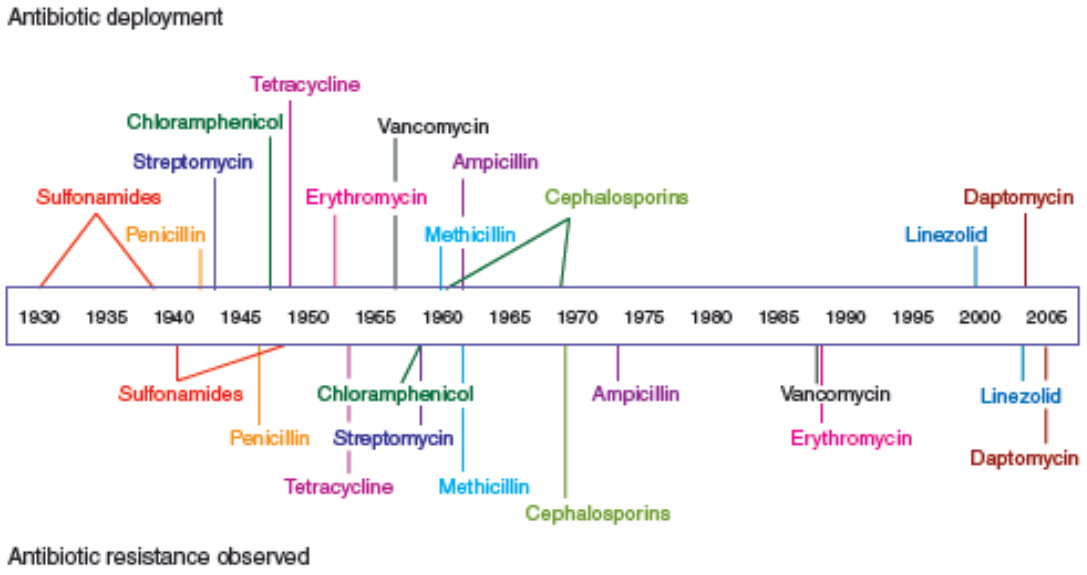

Figure 5.1 Timeline of antibiotic development and the evolution of resistance. The year each antibiotic was developed is depicted above the timeline, and the year resistance to each antibiotic was observed is depicted below the timeline (with the caveat that the appearance of antibiotic resistance does not necessarily imply that a given antibiotic has lost all clinical utility) (Clatworthy *et al.*, 2007)

The topic of antimicrobial targets and resistance mechanisms is an enormous area and it is not the intention in this thesis to cover it all. During this PhD study, I made repeated attempts to generate TDA resistant mutants, however, this was not possible. This led to an interest in the mechanism-of-action of TDA, and whilst the thesis work does give indications of this, the precise target was not identified. Therefore, the purpose of this chapter is to give a very brief introduction to the antibiotic field including antimicrobial peptide and biocides to present an overview of targets and resistance mechanisms. This knowledge and the experimental results that exist for TDA are combined in order to present a hypothesis on the mechanism-of-action of TDA.

5.1 Bacterial targets for antibiotics and other antimicrobials

Different antimicrobials can be used for combating bacteria and includes antibiotics, antimicrobial peptides and biocides. They differ in the way they are used and in their mechanism-of-action as described in the following sections. Most attention in this thesis has been given to antimicrobial peptides and biocides since preliminary experiments indicated that the activity TDA could be contributed to mechanisms resembling those two groups. TDA probably target the bacterial cell envelope, which is also the case for antimicrobial peptides. Due to difficulties getting resistant or tolerant strains, TDA could have multiple target sites like biocides.

5.1.1 Mechanism-of-action of antibiotics

Antibiotics are either natural products or synthetic chemicals used for treatment of bacterial infections in a host. They can also be used preventively in animal flocks for prophylaxis, as therapy for sick animals combined with prophylaxis in healthy individuals (metaphylaxis), and as growth promoters using the antibiotic at sub-therapeutic concentrations to increase body growth of the animal. The latter application is banned in countries within the European Union, however, it is still used in the US and Australia (Guardabassi *et al.*, 2006).

Antibiotics work by inhibition of biochemical pathways that are involved in biosynthesis of vital components in a bacterial cell, and the antibiotic attack a single biochemical target that is not present in the host. The major targets of antibiotics are (i) cell wall biosynthesis, (ii) protein biosynthesis, (iii) DNA replication and repair, and (iv) folate coenzyme biosynthesis (Figure 5.2) (Walsh, 2003b).

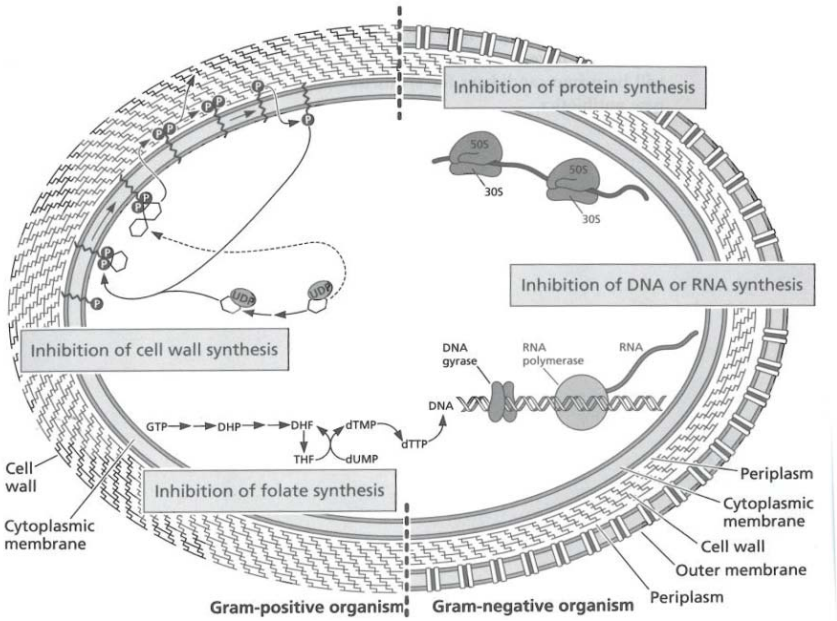

Figure 5.2 Major bacterial targets for antibiotics (Walsh, 2003b)

5.1.2 Mechanism-of-action of antimicrobial peptides

Antimicrobial peptides represent an evolutionary well-conserved group of bacterial inactivator molecules, and they are widespread in the innate immune systems of fungi, plants, insects and mammals (Yeaman & Yount, 2003). Five major classes of antimicrobial peptides exist: (i) anionic peptides, (ii) linear cationic α-helical peptides, (iii) cationic peptides enriched for specific amino acids (proline, glycine, histidine and tryptophan), (iv) anionic and cationic peptides containing cystein that form disulphide bonds, and (v) anionic and cationic peptide fragments of larger proteins (Brogden, 2005). Antimicrobial peptides have two possible applications in therapy. They can be used as direct antimicrobial agents due to their broad antibacterial spectrum and rapid action, or as immunomodulatory compounds in a new therapeutic approach as they stimulate the innate immune system (Hancock & Sahl, 2006).

Antimicrobial peptides excert their effect via several mechanisms. They attack many different microbial targets at the same time but with modest potency instead of blocking a specific high-affinity target (Peschel & Sahl, 2006). It is generally accepted that most antimicrobial peptides cause destabilization of the bacterial cell membrane exemplified by the Shai-Matsuzaki model (Figure 5.3). The peptide interacts with the membrane, followed by displacement of lipids and alteration of membrane structure and even collapse of the membrane (Zasloff, 2002).

Furthermore, formation of pores seems to be a central point in the peptide-membrane interaction. Pores allow passage of ions and small molecules, which lowers of proton gradient and destroy the membrane potential, stop ATP production and all cellular metabolism, finally leading to cell death (Huang, 2000). Some antimicrobial peptides also have intracellular targets (Table 5.1), e.g indolicidin and pleurocidin inhibit DNA synthesis and protein synthesis, respectively, (Subbalakshmi & Sitaram, 1998; Patrzykat *et al.*, 2002). In addition to the antimicrobial effect, some peptides also stimulate the immune system by boosting both innate and adaptive immune response which favours resolution of infections (Lai & Gallo, 2009). Hench, they are also referred to as host defence peptides.

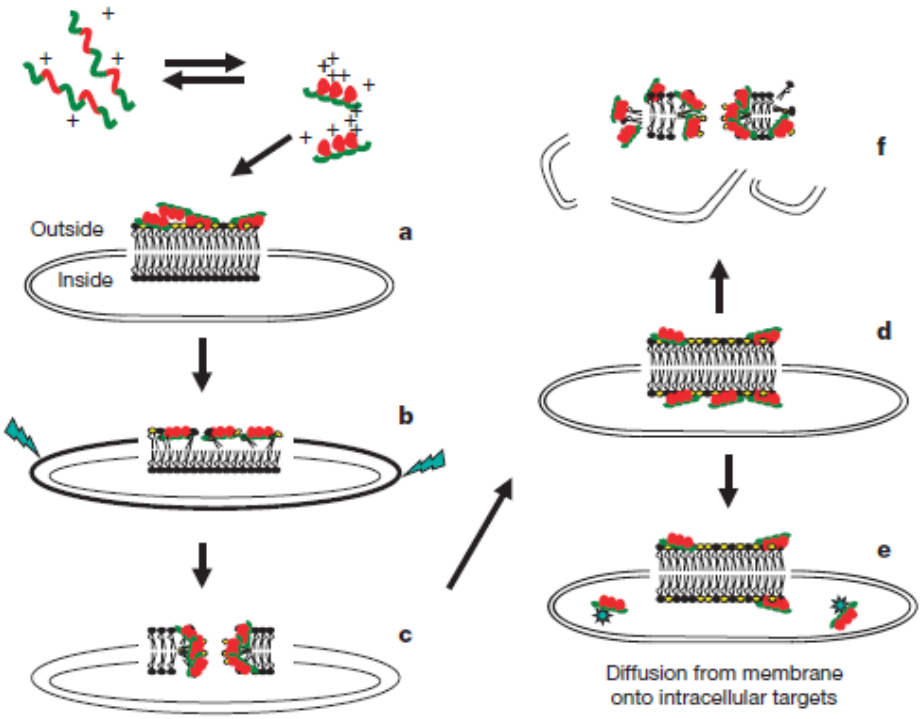

Figure 5.3 The Shai-Matsuzaki model for the mechanism of action of an antimicrobial peptide. An α-helical peptide is depicted. (a) Carpeting of the outer leaflet with peptides. (b) Integration of the peptide into the membrane and thinning of the outer leaflet. The surface area of the outer leaflet expands relative to the inner leaflet, resulting in strain within the bilayer (jagged arrows). (c) Phase transition and 'wormhole' formation. Transient pores form at this stage. (d) Transport of lipids and peptides into the inner leaflet. (e) Diffusion of peptides onto intracellular targets (in some cases). (f) Collapse of the membrane into fragments and physical disruption of the target cell's membrane. Lipids with yellow headgroup are acids, or negatively charged. Lipids with black headgroups have no net charge (Zasloff, 2002)

Table 5.1 Membrane and intracellular models of antimicrobial peptide killing and lysis. Modified from (Brogden, 2005)

Mode of intracellular killing	Examples of peptides
Flocculation of intracellular content	Anionic peptides
Alters cytoplasmic membrane septum formation	PR-39, PR-26, indolicidin, microcin
Inhibits cell wall synthesis	Mersacidin
Binds nucleic acids	Buforin II, tachyplesin
Inhibits nucleic acid synthesis	Pleurocidin, dermaseptin, PR-39, HNP-1, HNP-2, indolicidin
Inhibits protein synthesis	Pleurocidin, dermaseptin, PR-39, HNP-1, HNP-2, indolicidin
Inhibits enzyme activity	Histatins, pyrrhocoricin, drosocin, apidaecin

5.1.3 Mechanism-of-action of biocides

Biocides include chemicals (inorganic or synthetic organic molecules) with antiseptic, disinfectant and/or preservative activity (Chapman, 2003). They can be used for a variety of purposes on inorganic objects (hard surface disinfectants), externally on the skin to prevent or limit microbial infection (antiseptics and topical antimicrobials), for skin disinfection previous to operations or incorporated into pharmaceutical, cosmetics or other types of products (preservatives) to prevent microbial contamination (Russell, 2003). Often a product contains more than one biocide or active component.

In contrast to antibiotics, biocides generally act on a number of distinct targets with varied susceptibilities and the target may also occur in eukaryotic organisms (Gilbert *et al.*, 2002). One exception from this is triclosan (used in household products like toothpaste and soap) which inhibits the *fabI* gene encoding the enoyl-acyl carrier protein reductase of fatty acid biosynthesis (McMurry *et al.*, 1998). Biocides can be divided into four groups based on their mechanism-of-action (Table 5.2).

Table 5.2 Mechanisms-of-actions of biocides. Modified from (Chapman, 2003)

Mechanism-of-action	Group	Example of biocide
Electrophile biocides	Oxidants	Halogens
		Peroxy compounds
	Electrophils	Formaldehyde
		Isothiazolones
		Bronopol
		Inorganic ions (Cu, Hg, Ag)
Membrane active biocides	Lytic	Quaternary ammonium compounds
		Biguanides
		Phenols
		Alcohols
	Protonophores	Parabens
		Weak acids
		Pyrithione

Oxidants work directly via radical-mediated reactions to oxidize organic material (Clapp *et al.*, 1994; Dukan & Touati, 1996; Dukan *et al.*, 1999). Electrophils react covalently with cellular nucleophiles to inactivate enzymes and they might form intracellular free radicals which contribute to the lethal action (Collier *et al.*, 1990; Kimura & Nishioka, 1997). Lytic compounds cause destabilization of the bacterial membranes leading to cell lysis (Gilbert *et al.*, 1977; Chawner & Gilbert, 1989). Protonophores interfere with the ability of the cell membrane to maintain a proper

pH balance within the cell resulting in acidification of the interior and disruption of metabolism (Eklund, 1985).

5.1.4 How to study mechanisms-of-actions of antimicrobials

There are no "golden rules" describing how to study the mechanism-of-action of antimicrobials against bacteria, but such studies are normally initiated using basic microbiological analyses. Initially the spectrum of target organisms is often evaluated e.g. does it work against all bacteria (broad spectrum) or only some groups of bacteria (narrow spectrum). Then it is investigated if the compound is bacteriostatic or bactericidal to bacterial cell. Also, it is relevant to study if an antimicrobial agent causes disruption of the cytoplasmic membrane, which is often exemplified by leakage of intracellular components such as ATP, potassium, inorganic phosphate and materials absorbing at 260 nm (e.g pyrimidines and purines) (Lambert & Hammond, 1973; Ultee *et al.,* 2002; Raafat *et al.,* 2008; Gottlieb *et al.,* 2008; Nobmann *et al.,* 2010; Schneider *et al.,* 2010a). Electron microscopy can reveal if a compound causes visible damage to cells such as elongation or other shape malformations, membrane damage or complete lysis (Collier *et al.,* 1990; Bizani *et al.,* 2005; Raafat *et al.,* 2008).

The effect of antimicrobials on macromolecules can be studied by analyzing the incorporation of radioactive labeled precursors into cellular macromolecules while they are synthesized. For example isoleucin and histidies are incorporated into proteins, thymidine and uridine into nucleic acid, and glucoseamine are building blocks in synthesis of peptidoglycane (Patrzykat *et al.,* 2002; Schneider *et al.,* 2010a). Lack of such incorporation upon antimicrobial treatment point to a target in a biosynthetic pathway.

Bacterial cells express different genes as a result of environmental stimuli. By comparing gene expression patterns between bacteria exposed and not exposed to an antimicrobial compound, it may be possible to point at genes involved in the response and hence, potentially, to the mechanism-of-action of the drug. Transcriptomic analyses have been used to determine gene expression upon antimicrobial exposure (Hansen *et al.,* 2004; Brazas & Hancock, 2005; Raafat *et al.,* 2008; Jang *et al.,* 2008; O'Donnell *et al.,* 2009; Schneider *et al.,* 2010a), however, it can only be used on bacteria where commercial chips exist or on genome sequenced strains where custom made microarray chips can be prepared. These limitations in choice of strain can be circumvented by using e.g. RNA-Arbitrarily-Primed-PCR (Holmstrøm *et al.,* 2003; Papadimitriou *et al.,* 2008), however, this more limited analysis will not give the full picture of all genes. New sequencing methods such as 454 are not restricted to genome sequenced strains.

An alternative approach to reveal target(s) of an antibiotic include the use of biosensors, however, this method has not been published. The method is based on natural stress responses of bacteria to perturbation. These responses result in activation of specific genes which are monitored using reporter genes labeled with e.g. GFP variants. Thereby, the set of biosensors employed emit fluorescence of different wavelengths depending on the mode of the perturbation. Selective cell wall, ribosome and fatty acid biosynthesis can be identified as target of drugs in bacteria using this method (Dirk Bumann, University of Basel, personal communication).

5.1.5 Mechanism-of-action of tropodithietic acid

It is important from an applied point of view to know, which bacteria are affected by TDA and the mechanism-of-action of the compound. This understanding is required to determine e.g. the spectrum of use and to evaluate application possibilities. A collection of human pathogenic bacteria of both Gram-negative and Gram-positive bacteria were inhibited by TDA when determining the minimum inhibitory concentration (MIC) (Table 5.3). However, Gram-negative organisms were less sensitive to TDA as compared to Gram-positive bacteria. Other studies have also seen this broad-spectrum effect of thiotropocin (or TDA) for different human pathogens (Tsubotani *et al.,* 1984; Kintaka *et al.,* 1984).

The difference in sensitivity between Gram-negative and Gram-positive bacteria could be due to the different cell wall, assuming that the outer membrane of Gram-negative organisms could provide a protective shield against TDA. This is supported by the fact that only Gram-negative bacteria have increased sensitivity to TDA after treatment with EDTA (Porsby *et al.,* 2010). It has been reported that less thiotropocin (or TDA) is needed in order to reach the MIC value for a variety of different human pathogenic bacteria at low pH (Tsubotani *et al.,* 1984; Kintaka *et al.,* 1984). An explanation for this effect could be that the equilibrium of TDA at low pH values is moved to the protonated state resulting in a more lipophilic compound, which can more easily penetrate the outer membrane of Gram-negative bacteria as compared to the non-protonated form. However, this theory does not explain why the pH effect is also seen for Gram-positive organisms lacking the outer membrane. Similarly, Manson *et al.* (2006) found that a cationic peptide LAH4 only had bacteriolytic effect at acidic pH and not at neutral pH.

TDA is bactericidal against *Vibrio anguillarum* strain 90-11-287 (D'Alvise *et al.,* 2010). In this thesis work, similar observations have been found for both *Salmonella enterica* serovar Typhimurium (hereafter referred to as *Salmonella* Typhimurium) strain SL1344 and *Staphylococcus aureus* NCTC 12493 in both growth medium (LB broth) and in buffer (PBS) (Figure 5.4). TDA therefore has an effect on both growing and non-growing cells.

Table 5.3 MIC values (mg/l) of tropodithietic acid determined in LB broth. MIC values were determined in two independent experiments and if all gave the same value, this is stated in the table. For some strains, MIC varied a factor of two between experiments as indicated (Porsby *et al.*, 2010)

Gram	Species	Strain	MIC (mg/l)
Negative	*Salmonella* Typhimurium	SL1344 (wildtype)	625
	Salmonella Typhimurium	SL1344 (*ompF*::*aph*)	625
	Salmonella Typhimurium	SL1344 (*ompC*::*aph*)	625
	Salmonella Typhimurium	ATCC 14028s (wildtype)	625
	Salmonella Typhimurium	ATCC 14028s (Δ*tolC::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*acrB::Km*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*acrAB::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*acrD::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*acrEF::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*mdtABC::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*mdsABC::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*ermAB::Cm*^{R})	625
	Salmonella Typhimurium	ATCC 14028s (Δ*mdfA::Cm*^{R})	625
	Salmonella Typhimurium	ATCC 14028s (Δ*mdtK::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*macAB::Cm*^{R})	625
	Salmonella Typhimurium	L3 (Human pre-therapy clinical isolate)	625 - 1250
	Salmonella Typhimurium	L10 (Human post-therapy clinical isolate, *acr*AB+++)	625 - 1250
	Enterobacter cloacae	NCTC 10005	625 - 1250
	Serratia marcescens	NCTC 2847	625 - 1250
	Klebsiella pneumoniae	NCTC 10896	625 - 1250
	Klebsiella pneumoniae	NCTC 9633	625
	Escherichia coli	NCTC 10538 (K12)	155 - 625
	Morganella morganii	NCTC 235	155
	Pseudomonas aeruginosa	NCTC 10662	625
	Vibrio anguillarum	90-11-287 (from rainbow trout)	40 - 80
	Phaeobacter ssp.	27-4 (from Spanish turbot unit) (wildtype)	310
	Phaeobacter ssp.	JBB1001 (*tdaB*::EZ-Tn5,Kan)	155
Positive	*Staphylococcus aureus*	NCTC 8532 (MSSA)	155
	Staphylococcus aureus	NCTC 12493 (MRSA)	155
	Enterococcus faecalis	NCTC 775	80
	Enterococcus faecalis	NCTC 7171	80
	Listeria monocytogenes	NC12427	80

Salmonella Typhimurium: *Salmonella enterica* serovar Typhimurium

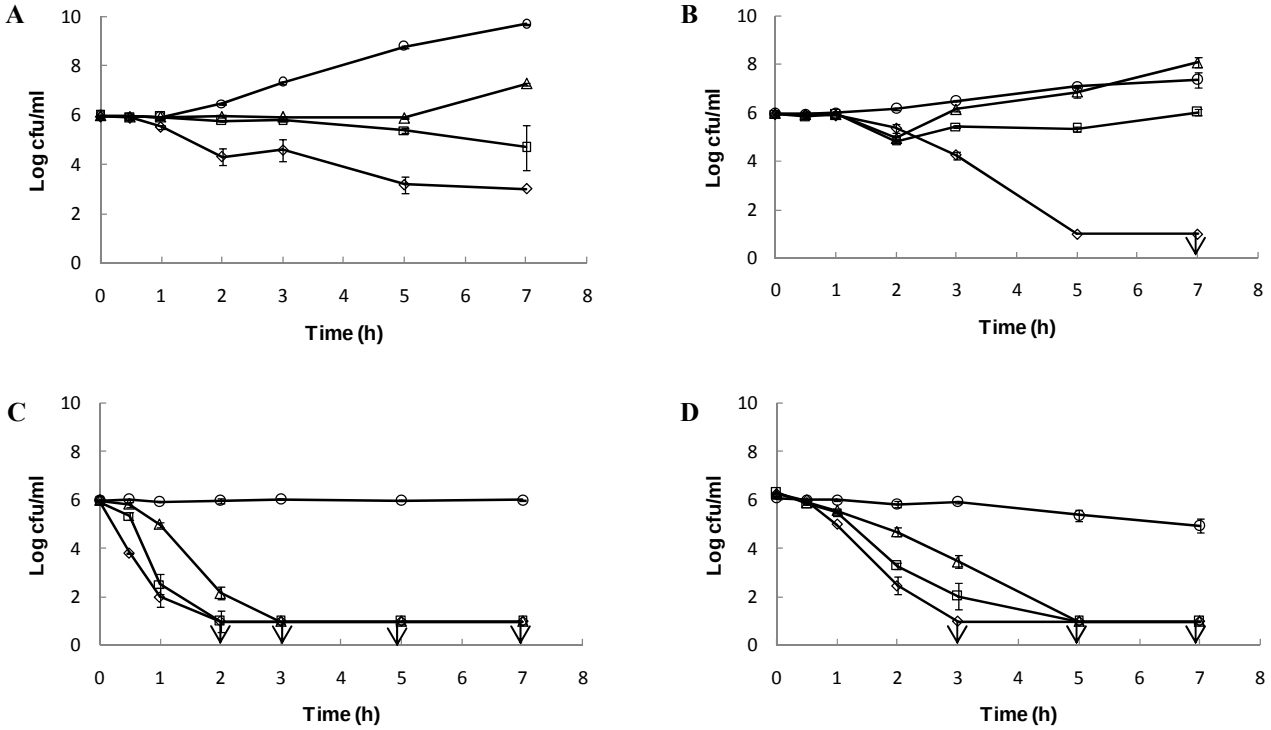

Figure 5.4 Killing kinetics of tropodithietic acid (TDA) on *Salmonella enteric* serovar Typhimurium strain SL1344 (A and C) and *Staphylococcus aureus* stain NCTC 12493 (B and D) in LB medium (A and B) and in phosphate buffered saline (C and D). The following concentrations of TDA were used: MIC (◇), ½MIC (□), ¼MIC (△) and a growth control containing no TDA (○). Counts are mean of duplicates and error bars represent standard deviations. Arrows indicate observations below detection limit (Porsby *et al.*, 2010)

Salmonella Typhimurium mutants with non-functional porins or efflux pumps were approximately as sensitive to TDA as their parent strains (Table 5.3). This indicates that these structures are not involved in the innate resistance to TDA in *Salmonella* Typhimurium. Also, TDA does not cause any visible damage to *Escherichia coli* strain K12 cells (Figure 5.5). Kintaka *et al.* (1984) did not see morphological changes to *Proteus mirabellis* IFO 3168, but very poor lysis was observed in *E. coli* NIHJ JC2 after exposure to high concentrations of the tautomeric form of TDA, thiotropocin.

Keeping in mind that TDA works very well on non-growing cells these results could collectively indicate that TDA does not have to enter the interior of a bacterial cell, i.e. TDA does not have to cross the cell wall or the membrane, in order to exert its effect. It is therefore plausible that TDA targets the cell envelope, and it must be a component that both Gram-positive and Gram-negative bacteria have in common as TDA is antibacterial against both groups. Consequently, it is in this thesis suggested that TDA targets either the peptidoglycan or the cytoplasmic membrane. The target of TDA in *Salmonella* Typhimurium has been studied using a biosensor approach (see section 5.1.4), and the results indicate that the cell envelope and perhaps the peptidoglycan structure is the target (Dirk Bumann, University of Basel, personal communication).

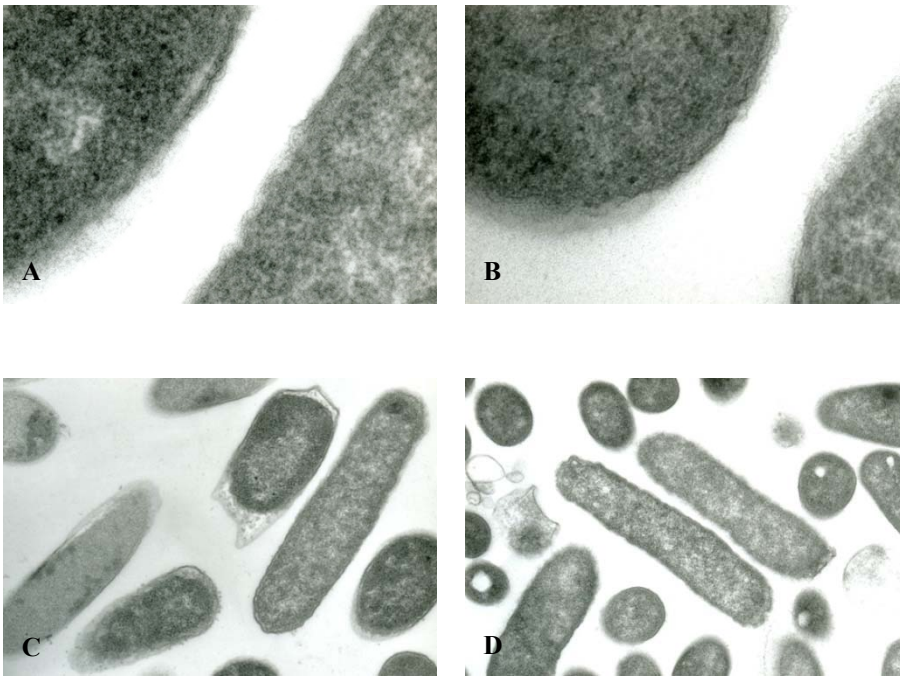

Figure 5.5 Transmission electron microsgraphs of *Escherichia coli* strain K12 treated (A and C) and not treated with (B and D) tropodithietic acid. Magnification: 150,000x (A and B) and 20,000x (C and D). Porsby, unpublished data

The cell wall including the peptidoglycan posses well known targets for antibiotics. There are three main phases in cell wall biosynthesis which all are targets for antibiotics (van Heijenoort, 2001(#379)):

- First phase (cytoplasmic phase): The structural unit of peptidoglycan layers, muramyl pentapeptide, is synthesized in the cytoplasma
- Second phase (membrane-associated phase): Muramyl pentapeptide is bound to the cytoplasmic surface of the cell membrane and translocated to its external face through formation of two lipid intermediates
- Third phase (extracytoplasmic phase): Glycine and peptide strands of muramyl pentapeptide are cross-linked by membrane-bound transglycolase and transpeptidase, respectively

The beta-lactams (e.g. penicillin, cephalosporin, carbapenems, and monobactams) is effective against both Gram-positive and Gram-negative bacteria and exert its effect on the third phase. These antibiotics contain a beta-lactam ring which is recognized by the active site of penicillin-binding proteins involved in crosslinking glycan chains through transpeptidase reactions or removal of excess terminal D-alanine after completion of the crosslinking process (Schneider & Sahl, 2010b). Antibiotics that act on the second phase in the cell wall synthesis include the group glycopeptide (e.g. vancomycin and teicoplanin). They cannot penetrate the pores in the outer

membrane of Gram-negative bacteria and are therefore only active against Gram-positive organisms (Walsh, 2003a). The activity of glycopeptides are due to binding of the antibiotic to the D-alanine-D-alanine terminus of the peptide side chain of lipid II (Schneider *et al.,* 2010b). This prevents incorporation of the D-alanine-D-alanine terminus into growing peptidoglycan. The first phase in the synthesis is inhibited by the antibiotic D-cycloserine by inhibition of the enzymes alanine racemate and D-alanine-D-alanine ligase which is involved in incorporation of alanine into cell wall precursors (Lambert & Neuhaus, 1972). It is unknown, which of the three steps in the biosynthesis of peptidoglycan that TDA targets.

As part of this PhD study, a series of microarray experiments on *Salmonella* Typhimurium strain SL1344 exposed to TDA were conducted, however, I am currently waiting for the results. This expression study could indicate which genes are affected by TDA exposure, and hence, potentially point to the mechanisms-of-action.

5.2 Antimicrobial resistance and tolerance

Resistance in bacteria against virtually all antibiotics have been seen within years of usage (Clatworthy *et al.,* 2007), and different mechanisms depending on the type of antibiotic have been developed. Lately, the term "tolerance" to a drug has been introduced which also describes the ability of a cell to withstand otherwise lethal concentrations of a antibiotic. However, tolerance does not involve genetic changes in the bacteria (Lewis, 2007).

5.2.1 Definition of resistance and tolerance

Resistance is a relative term mainly used for antibiotics. According to the microbiological definition, a strain is considered resistant if it grows in the presence of higher concentrations of the antibiotic as compared to phylogenetically related strains. On the other hand, a strain is considered clinically resistant if it survives antimicrobial therapy (Guardabassi *et al.,* 2006). Determining the MIC value is one way to investigate if a strain is resistant, intermediate or susceptible to a antibiotic. The MIC value is the lowest concentration of an antibiotic that under *in vitro* conditions inhibits visible growth of the bacteria within a defined period of time (Andrews, 2001; Andrews, 2006). To determine if a strain is resistant, the MIC value has to be compared to either microbiological or clinical breakpoints. Microbiological breakpoints are based on the MIC distribution for a bacterial species, while clinical breakpoints are not only considering the MIC distribution for a species but also *in vivo* parameters such as bacterial distribution in the host, and pharmacokinetics and pharmadynamics of the drug (Guardabassi *et al.,* 2006). The clinical dose of antibiotic are near the MIC value, and resistant strains will therefore lead to treatment failure of the bacterial infection.

Many articles and reviews deal with the topic of resistance to antimicrobial peptides, but I have not been able to find a definition of resistance to antimicrobial peptides. However, keeping in mind that they are to be used as direct antimicrobials agents for combating infections, the same definitions and reflections as for antibiotics (microbiological and clinical) can be proposed for antimicrobial peptides.

In respect to biocides, the term resistance is not well-defined. The definition proposed by Russell (2003) resembles the microbiological definition for antibiotics: "*A culture is considered to be resistant to a biocide when it is not inactivated by an in-use concentration of biocide, or a biocide concentration that inactivates other strains of that organism*". On the other hand, Chapman (2003) use the MIC value to define resistance as "*a measurable increase in the MIC value, often by a factor of 4 – 16-fold*". If using a MIC based definition, one should bear in mind that the in-use concentration of e.g. disinfectants is much higher that the MIC value, therefore, a resistant strain will probably not survive the disinfectant process (Bloomfield, 2002).

A stage between susceptible and resistant exists for antibiotics where a strain can tolerate increased concentrations of the antibiotic but not as high concentrations as if the same strain had developed resistance. Lewis (2007) defined tolerance as "*the ability of cells to survive killing by antibiotics without expressing or using resistance mechanisms*". A similar stage is likely also to exist for antimicrobial peptides and biocides.

5.2.2 Resistance mechanisms for antibiotics

Bacteria have many different mechanisms to overcome the effect of antibiotics (Table 5.4). Most common are enzymatic drug inactivation, modification or replacement of drug target, efflux of drug using pumps, and reduced drug uptake. Protection of target and drug trapping are not as common.

Resistance to antibiotics can be divided into intrinsic and acquired resistance (Guardabassi *et al.,* 2006). Intrinsic resistance is caused by a structural or functional trait which gives all members of a bacterial group resistance against a particular drug or a class of antibiotics. An example is the resistance of Gram-negative bacteria to glycopeptides (e.g. vancomycin and teicoplanin) as the drug cannot penetrate the outer membrane (Schneider *et al.,* 2010b). Acquired resistance is only associated with some strains within a genus or species, and it is due to a genetic change in the bacterial DNA caused by either a mutation or horizontal acquisition of foreign genetic material. For example chromosomally acquired streptomycin resistance is frequently due to mutations in the gene *rpsL* encoding the ribosomal protein S12 (Nair *et al.,* 1993; Honoré & Cole, 1994; Olkkola *et al.,* 2010), whereas resistance to methicillin is associated with acquisition of a SCC*mec* mobile genetic element, which contains the *mecA* resistance gene (Okuma *et al.,* 2002). Acquired resistance is

heritable due to multiplication of bacterial cells, and the resistance will be carried on to the next generations.

Table 5.4 Mechanisms of bacteria leading to resistance against antibiotics

Mechanism of resistance	Effect	Example of drug	Reference
Enzymatic drug inactivation	Modify active nucleus of drug leading to inability of drug to bind to target site	Beta-lactams Aminoglycoside	(Knox *et al.,* 1996) (Jana & Deb, 2006)
Modification or replacement of drug target	Target is structurally modified or replaced so drug can not bind	Glycopeptides Quinolones MLS	(Al-Obeid *et al.,* 1990) (Jalal & Wretlind, 1998) (Pernodet *et al.,* 1996)
Efflux pumps	Reduced concentration of drug in cytoplasm	Tetracyclines Ciprofloxacin	(Li *et al.,* 1994) (Piddock *et al.,* 2000)
Reduced drug uptake	Loss, reduced size, or decreased expression of porins leading to less drug inside the cell	Beta-lactams Fluoroquinolones	(Pages *et al.,* 2009) (Danilchanka *et al.,* 2008)
Target protection	RPPs bind to ribosomes in proximity to drug binding site and together with conformational changes drug can not bind	Tetracyclines	(Connell *et al.,* 2003)
Drug trapping	Increased production of drug target or another molecule with affinity for drug leading to reduction of free drug at target site	Vancomycin	(Cui *et al.,* 2000)

MLS: Macrolide-lincosamide-streptogramin type B RPPs: Ribosomal protection proteins

5.2.3 Tolerance to antimicrobials

Tolerance is another way to evade the effect of antimicrobials as bacteria can develop tolerance to antibiotics, antimicrobial peptides and biocides by adapting to harsh conditions which might otherwise kill them. Tolerance is a temporary state, and it is not due to mutations or acquisition of external DNA as it is the case for 'traditional' antibiotic resistance. More likely it is a transient change in gene expression which provokes a physiological state resulting in the ability to tolerate and survive otherwise harmful concentrations of the drug or compound (Miller *et al.,* 2004). Therefore, tolerance is a non-heritable phenotype.

The phenomenon of persister cells is an example of phenotypic heterogeneity where a bacterial population do not act uniformly. When the population is treated with antibiotic, a small fraction of bacteria survives and when the antibiotic is removed, these cells re-grow as a new population that is just as sensitive to the antibiotic (Bigger, 1944; Lewis, 2007; Gefen & Balaban, 2009). This reversible tolerance has even been called "a phenotypic switch" (Balaban *et al.,* 2004). Very little knowledge exists on the molecular mechanism underlying this tolerance, however, the *hipA* gene,

which may encoded a toxin protein that promote dormancy, is known to be involved in development of persister cells in *E. coli* (Moyed & Bertrand, 1983; Schumacher *et al.,* 2009).

5.2.4 Lack of resistance and tolerance to tropodithietic acid

If TDA producing bacteria are to be used in aquaculture it is important to evaluate if resistance or tolerance can evolve. A collection of human and fish pathogens strains (Table 5.3 page 44, excluding the mutants) were exposed to purified TDA (Porsby *et al.,* 2010). A range of different methods were applied in order to select for resistant mutants including single exposure to TDA administered via different methods. However, no TDA resistant mutants were selected. Tolerant strains were selected by multiple exposures to subinhibitory concentrations of TDA. The approach has proven especially successful for antimicrobial peptides and biocides (Méchin *et al.,* 1999; Suller & Russell, 1999; Perron *et al.,* 2006; Karatzas *et al.,* 2007), and it resulted in two-fold increased MIC values. However, the effect was abolished after only one passage in fresh medium, indicating a reversible tolerance. Sallum & Chen (2008) reported the same rapid reverting of the MIC value to the antimicrobial peptide cecropin B for *V. anguillarum* and *Vibrio vulnificus*.

The fact that it was not possible to select TDA resistant mutants could indicate that more than one mutation or genetic change in the bacterial genome is needed in order to gain resistance to TDA. This is also the case for antimicrobial peptides and biocides (Russell, 2003; Peschel *et al.,* 2006). However, a large number of mutations may be detrimental to the bacterial cell, and hence, not likely to co-occur. The transient, slightly increased tolerance to TDA is most likely caused by a phenotypic switch caused by e.g. altered gene expression.

5.2.5 Self-protection of tropodithietic acid producing strains

Many bacteria produce antibiotics that potentially could be toxic to themselves (Martín *et al.,* 2005). Typically, organisms producing antibiotics have evolved resistance mechanisms for protection against their own drug. The mechanisms for self-protection include (Cundliffe, 1991):

- Development of membrane permeability barriers coupled with efficient efflux mechanisms for removal of drug molecules from the cells
- Inactivation or sequestration of intracellular drug molecules and any biologically active precursors thereof
- Modification or replacement of the target site(s) at which specific drugs normally act

Genes involved in biosynthesis of an antibiotic is often organized in a clusters, and close to these clusters genes encoding resistance to the antibiotic or genes encoding efflux pumps or other transporter systems can be found (Martín *et al.,* 2005). In some *Streptomyces* species, ABC

transporters are involved in pumping the antibiotic out of the producer strain (Rodríguez *et al.,* 1993; Linton *et al.,* 1994). there are many ABC transporter genes in the proximity of genes encoding the biosynthesis of TDA in *Phaeobacter* and *Ruegeria* strains (*tdaABCDE* and *tdaF*). An ABC transporter system is located just before the *tdaA* gene in *Phaeobacter gallaeciensis* strain SK2.10 and BS107, and a transcriptional regulator, LysR-like, is located upstream the transporter genes. In addition, two ABC transporter genes are localized between *tdaE* and *tdaF*, but no transporter genes are found after *tdaF*. This could indicate that these ABC transporters systems are involved in protection of *Phaeobacter* strains against TDA.

In this work, it was found that the TDA producer strain *Phaeobacter* strain 27-4 was not protected against TDA (Porsby *et al.,* 2010). This could be due to the conditions that were used for determining the MIC value, as it was determined after incubation for two days. TDA is produced in the late exponential growth phase, and perhaps a self-protection-mechanism such as ABC transporters is only expressed when TDA is beeing produced. Geng & Belas (Geng *et al.,* 2010) suggested that TDA functions as an autoinducer of its own biosynthesis, however, it can also be speculated if TDA induce expression of the ABC transporter systems through activation of the *LysR* gene. Thereby these transporters are only expressed when TDA is being produced.

TDA could also be inactivated in the producer cell as a part of the biosynthesis e.g. by binding another compound to the TDA molecule. This TDA containing molecule, which are not lethal to the producer strain, are then exported out of the cell by the ABC transporters. Finally, TDA and the in-activator molecule are separated and TDA now have antibacterial activity. Enough is not known about the biosynthesis of TDA to confirm or reject this theory.

5.3 Conclusions from chapter 5

Antimicrobials such as antibiotics, antimicrobial peptides and biocides have different mechanisms-of-actions. TDA, which is bactericidal against both Gram-negative and Gram-positive bacteria, probably targets the bacterial cell envelope, likely the peptidoglycan. This is a well-known target for some antibiotics (e.g. β-lactams and glycopeptides), but also antimicrobial peptides attack the cell wall. Strains with resistance or tolerance to TDA were very hard to select, and this could indicate that TDA have multiple target sites in a bacteria which it is the case for biocides. Also, the slight increase in tolerance was not stable. Therefore, it is hypothesized that the transient increase in TDA tolerance is due to a phenotypic switch caused by e.g. altered gene expression. The fact that resistance and enhanced tolerance is difficult to obtain, is promising from an application point of view. It is not likely that the use of TDA producing bacteria as fish probiotic will lead to TDA resistance among fish pathogenic bacteria, thereby, the probiont will not lose its disease preventing effect due to decreased sensitivity among the fish pathogenic bacteria.

6 Concluding remarks and future perspectives

The aquaculture sector is expanding rapidly to the benefit of the growing world population. However, bacterial diseases are important constraints to this growth. Today some of these diseases are controlled with antibiotics, however, due to fear of development of resistant bacteria and spread of this resistance among bacteria in the food chain, alternatives to antibiotics are needed. Probiotic organisms, which are "*live organisms which when administered in adequate amounts confer a health benefit on the host*" (FAO/WHO, 2001), can be the solution in some cases e.g. in marine larvae cultures. Such probiotic organisms could be bacteria belonging to the *Roseobacter* clade.

Roseobacter clade members are common in marine environments as both non-culturable clones and easily culturable isolates. In natural settings, they are often associated with surfaces or particles indicative of a preference for an attached lifestyle. The *Roseobacter* clade species *Phaeobacter gallaeciensis*, *Phaeobacter inhibens* and *Ruegeria mobilis* colonize turbot rearing facilities in both Denmark (this thesis) and Spain despite the very different water sources (the Galician Atlantic Ocean opposed to the Danish fjord Limfjorden). Members of the clade can also be found in aquaculture settings of e.g. haddock, cod and different molluscs such as scallops, oysters and clams.

The success of *Roseobacter* clade bacteria in natural environments and in aquaculture can potentially be explained by their ability to produce antagonistic compounds, which is assumed to give them an advantage over other organisms. The species *Ph. gallaeciensis*, *Ph. inhibens* and *R. mobilis* inhibit or kill a variety of fish pathogenic and marine bacteria, probably due to the antagonistic effect of the secondary metabolite tropodithietic acid (TDA). However, at the same time they are not lethal to egg yolk sac larvae. The fact that these bacteria are naturally occurring in aquaculture and seem to be superior to other bacteria makes them promising probiotic candidates. One way to reveal if TDA is causing a probiotic effect would be to conduct *in vivo* experiments where e.g. larvae are infected with a pathogen and treated with a TDA producing strain (wildtype) or a knockout mutant deficient of TDA production.

Production of TDA is influenced by environmental parameters such as temperature and presence of marine salts, but also by the physical environment. Work in this thesis show that *Ruegeria* strains only produce TDA when grown at stagnant conditions, whereas *Phaeobacter* strains produce the compound both at shaking and stagnant conditions. A brown pigment, the ability to inhibit *Vibrio anguillarum* strain 90-11-287 and growth in rosette-like structures co-occur with TDA production. To the best of my knowledge, the only exception is strain 27-4 which based on 16S rRNA and *gyrB* phylogeny is identified as a *Phaeobacter*, but phenotypically it behaves like the *Ruegeria* strains.

The ability to grow in rosettes and the production of TDA always seem to co-occur in both *Phaeobacter* and *Ruegeria* species. Furthermore, the brown pigment is produced in the top layer of a stagnant culture which is also where the rosette-formation is observed. Therefore, it would be interesting to investigate if TDA production only takes place in cells that grow in rosettes and not in planktonic cells, and if the phenotype is mediated by the physical conditions at the air-liquid interphase.

The biosynthesis of TDA is not fully understood, and only some of the genes involved have been identified. It is still unknown e.g. how the two sulfur molecules are added to TDA. The genes *tdaABCDEF* are essential to biosynthesis of TDA and they have been identified in all genome sequenced strains which produce TDA. In *Ruegeria* strain TM1040 they are located on a plasmid, whereas in *Phaeobacter* strain BS107 and SK2.10 they are found on the chromosome. From an applied point of view, it is an advantage to use a strain with the "producer genes" located on the chromosome, as plasmids can be lost spontaneously leading to a probably non-effective probiont.

In the present thesis, it was shown that TDA is bactericidal to both Gram-negative and Gram-positive bacteria, however, the Gram-negative bacteria are generally less sensitive to the compound. TDA is also bactericidal to non-growing cells. It is suggested that TDA does not have to enter the bacterial cell to reach its target, and therefore, the cell envelope might contain the target site(s) for TDA. This is supported by the biosensor results that also pointed to the cell envelope as target and perhaps the peptidoglycan structure.

It was not possible in this PhD study to obtain TDA resistant mutants which can indicate that TDA has multiple target sites in or on the bacterial cell, hence, resistance requires a multitude of mutations that are unlikely to co-occur. Only low levels of tolerance could be induced by multiple exposures to subinhibitory concentrations of TDA. However, the decreased sensitivity to TDA was abolished after one passage without the compound. This transient tolerance might be due to a phenotypic switch which is rapidly reversed. This switch phenomenon may be caused by e.g. altered gene expression and is not due to changes in the DNA.

It is hypothesized that TDA is also produced in natural setting (e.g. aquaculture) but it is unknown at which concentrations. It is plausible that fish pathogenic bacteria in a fish tank will experience subinhibitory concentrations of TDA upon use of a TDA producing bacteria as probiont. Subinhibitory of antibiotics is known to cause altered virulence gene expression in other bacteria (Weir *et al.,* 2008; Nanduri *et al.,* 2009). From an application point of view, it would be important to know if subinhibitory concentrations of TDA influence the virulence of fish pathogenic bacteria.

TDA producing bacteria have been added to fish larvae and also co-cultured with algae and do not have adverse effects on neither of the eukaryotes (D'Alvise, unpublished data). However, any side effects of long term exposure of eukaryotes to TDA need to be investigated.

Assuming that TDA is not toxic, the broad spectrum of activity of TDA also evokes interest in the compound as an antibiotic with broader use, e.g. in humans. TDA is unstable at 30ºC and above (Bruhn *et al.,* 2005), and this could be either advantageous ensuring a rapid turnover or a drawback hampering efficient use.

Members of the *Roseobacter* clade are abundant in several natural marine environments and they seem to prefer a surface attached lifestyle. Some species within the *Roseobacter* clade grow in rosette-like structures, and this phenotype co-occurs with increased attachment ability. It can be speculated if this rosette-state triggers TDA production. The cells are in a state where they are ready to attach to a surface, and they need TDA as "a weapon" to remove other attached bacteria to get a spot on the surface.

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Paper 1

Cisse Hedegaard Porsby, Kristian Fog Nielsen and Lone Gram (2008)

Phaeobacter and *Ruegeria* species of the *Roseobacter* clade colonize different niches in a Danish turbot (*Scophthalmus maximus*) rearing farm and antagonize turbot larval pathogens under different growth conditions

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Phaeobacter and *Ruegeria* Species of the *Roseobacter* Clade Colonize Separate Niches in a Danish Turbot (*Scophthalmus maximus*)-Rearing Farm and Antagonize *Vibrio anguillarum* under Different Growth Conditions▽

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Members of the *Roseobacter* clade colonize a Spanish turbot larval unit, and one isolate (*Phaeobacter* strain 27-4) is capable of disease suppression in in vivo challenge trials. Here, we demonstrate that roseobacters with antagonistic activity against *Vibrio anguillarum* also colonize a Danish turbot larval farm that relies on a very different water source (the Danish fiord Limfjorden as opposed to the Galician Atlantic Ocean). Phylogenetic analyses based on 16S rRNA and gyrase B gene sequences revealed that different species colonized different niches in the larval unit. *Phaeobacter inhibens*- and *Phaeobacter gallaeciensis*-like strains were primarily found in the production sites, whereas strains identified as *Ruegeria mobilis* or *Ruegeria pelagia* were found only in the algal cultures. *Phaeobacter* spp. were more inhibitory against the general microbiota from the Danish turbot larval unit than were the *Ruegeria* spp. *Phaeobacter* spp. produced tropodithietic acid (TDA) and brown pigment and antagonized *V. anguillarum* when grown under shaking (200 rpm) and stagnant (0 rpm) conditions, whereas *Ruegeria* spp. behaved similarly to *Phaeobacter* strain 27-4 and expressed these three phenotypes only during stagnant growth. Both genera attached to an inert surface and grew in multicellular rosettes after stagnant growth, whereas shaking conditions led to single cells with low attachment capacity. Bacteria from the *Roseobacter* clade appear to be universal colonizers of marine larval rearing units, and since the Danish *Phaeobacter* spp. displayed antibacterial activity under a broader range of growth conditions than did *Phaeobacter* strain 27-4, these organisms may hold greater promise as fish probiotic organisms.

Bacteria from the *Roseobacter* clade are widely distributed in marine environments (37, 51), and this group includes 38 different genera (7). The organisms constitute 20 to 30% of prokaryotes in 16S rRNA gene libraries from surface water (11). Even though the clade is detected often, little is known about the species distribution and biogeography in the oceans (7) and in more closed environments like fish farms using oceanic water.

Some members of the *Roseobacter* clade produce secondary metabolites (23, 31), and the production of, e.g., antimicrobial compounds may contribute to their dominance in several niches. Also, the secondary metabolite production has caused interest in the *Roseobacter* clade from a biotechnological perspective. For instance, *Phaeobacter gallaeciensis* (formerly *Roseobacter gallaeciensis*) (35) and *Phaeobacter inhibens* are antagonistic against bacteria such as *Vibrio anguillarum*, *Vibrio splendidus*, *Vibrio cholerae*, *Bacillus subtilis*, *Halomonas* spp., and *Pseudoalteromonas* sp. due to production of tropodithietic acid (TDA) (6, 8, 9, 28). The antagonism against *Vibrio* species and the association of some roseobacters with algae (1, 11, 22) have spurred an interest in *P. inhibens* as a possible fish probiotic organism, as algae are typically used as live feed in marine larval rearing. Probiotics have been defined by FAO/WHO (17) as "live microorganisms which when administered in adequate amounts, confer a health benefit on the host." Indeed it has been demonstrated that the survival of scallop, bream, and turbot larvae can be increased by adding cell extracts of roseobacters to the tank water or feeding the larvae with rotifers loaded with the probiont (34, 39, 44).

The *Roseobacter* clade is one of the dominant groups of colonizers on surfaces in marine environments (13), and in a Spanish turbot larval unit roseobacters were found more often on surfaces (e.g., tank walls) than in the water and a number of specific subtypes appeared as stable colonizers of the rearing unit (29). Specifically, for the *Phaeobacter* strain 27-4, which was isolated from a Spanish turbot farm, it was demonstrated that production of the anti-*Vibrio* substance TDA occurred only under growth conditions that also facilitated biofilm formation at the air-liquid interface and on inert surfaces (8, 9).

With the rapidly growing aquaculture industry now supplying more than 40% of the fish used for human consumption (16), there is an intense interest in disease control measures that do not rely on classical antibiotics. The roseobacters, as mentioned, appear to be one such option as fish probiotics. However, it is not known if their colonization of the Spanish turbot larval unit is a unique finding or if the clade in general due to its association with algae and its dominance in the

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marine environment will be a common component of the microbiota in marine fish-rearing units and, hence, a more universal fish probiotic candidate.

The purpose of the present study was to determine if isolates belonging to the *Roseobacter* clade also were selected in a turbot larval farm using a water source (water from a Danish fiord) very different from the Atlantic marine waters used in Galician turbot-growing facilities. In the model organism, *Phaeobacter* strain 27-4, anti-*Vibrio* activity is tightly coupled with specific (stagnant) growth conditions, and the purpose of the present study was also to determine if this is a general trait in *Roseobacter* clade strains that produce TDA. From an applied fish-farming perspective, it would be advantageous to select probiotic strains where anti-*Vibrio* activity occurred under a broad range of growth conditions.

MATERIALS AND METHODS

Bacterial strains. The target strain used for testing antimicrobial activity of bacteria from the turbot-growing unit was *V. anguillarum* 90-11-287 (serotype O1) isolated from rainbow trout (46). *Roseobacter* clade strains 27-4, 8-1, 234-2, 234-9, 234-10, 256-7, 267-1, 270-3, and 632-1 were isolated from two Spanish turbot-rearing units due to their antimicrobial properties (28). *Silicibacter* sp. strain TM1040 originates from dinoflagellate cultures of *Pfiesteria piscicida* (36). All strains were stored at −80°C in a freeze medium (30.0 g tryptone soy broth [Oxoid CM129B; Oxoid, Hampshire, England], 5.0 g glucose, 20.0 g skim milk powder, 40.0 g glycerol, 1000 ml H_2O) (21). Strains were cultured on marine agar 2216 (MA; Difco, BD, Sparks, MD) or in marine broth 2216 (MB; Difco) for 2 to 3 days at 25°C.

Sampling and isolation of antimicrobial strains. Samples were taken for microbiological analysis from a Danish turbot larval farm in October 2006. The turbot unit used water from Limfjorden, which is a shallow fiord transecting the Danish Jutland peninsula, and the salinity is around 2.5%. Samples were collected from walls and water from fish tanks; tanks with rotifers, nauplii, and zooplankton; and bags with algal cultures. Sterile gloves, swabs, and plastic beakers were used for sampling. All samples were 10-fold diluted in sterile seawater and spread onto MA. Liquid samples were diluted directly, whereas swab samples were mixed thoroughly with 1 ml autoclaved seawater (constituting a 10^0 dilution.). MA plates were incubated for 6 days at 20°C. To isolate colonies with antimicrobial activity, MA plates were replica plated onto seawater agar (1,000 ml seawater, 3.33 g Casamino Acids [Difco 223050], 20 ml 20% glucose, and 10 g agar) plates containing 5×10^5 to 1×10^6 CFU of *V. anguillarum* 90-11-287/ml agar. The agar was held at 44°C and mixed with *V. anguillarum* 90-11-287 previously grown in 10 ml MB for 2 days at 25°C. Colonies causing clearing zones after incubation of the seawater agar plates for 24 h at 25°C were isolated from the original MA plate and pure cultured. All strains were tested in a spot inoculation test and a well diffusion assay using colony mass and sterile-filtered supernatants, respectively, to verify the antimicrobial activity. In both assays, the agar was seeded with *V. anguillarum* 90-11-287 as described above, and *Phaeobacter* strain 27-4 was used as a positive control. Colonies of strains grown on MA at 25°C for 3 days were inoculated in one spot (approximately 0.5 cm) on seawater agar or Instant Ocean agar (1,000 ml H_2O, 30.0 g Instant Ocean [Aquarium Systems Inc., Sarrebourg, France], 3.33 g Casamino Acids, 20 ml 20% glucose, and 10 g agar). The clearing zone around the spot was read after 24 and 48 h at 25°C. For the well diffusion assay, 70 μl sterile-filtered (pore size, 0.22 μm; Millipore, Bedford, MA) supernatants of each isolate grown in 10 ml MB in 25-ml bottles for 3 days at 25°C under stagnant or shaking (200 rpm) conditions were added to wells (diameter, 6 mm) in solidified seawater agar. Diameters of clearing zones were measured after 24 h at 25°C.

Sampling and isolation of strains representing the general microbiota. To determine if the *Roseobacter* clade bacteria would have a pronounced effect on the general microbiota of the turbot larval farm, we investigated the ability of the *Roseobacter* clade strains to inhibit representative strains of the microbiota. Plate counts on six samples collected from the Danish turbot farm in January and February 2008 from three tanks (water and surfaces) were performed as described above. Colonies were isolated randomly and pure cultured. Spot assays were performed with 17 isolates embedded in Instant Ocean agar, and the *Roseobacter* clade strains were spotted on top of it as described above. *Phaeobacter* strain 27-4 was also tested against the 17 isolates. The 17 isolates were identified by biochemical tests and BLASTN (2) search on the National Center for Biotechnology Information (NCBI) database (4) (≥98% identity) using 16S rRNA sequences (for biochemical tests and sequencing procedures, see below).

Identification of bacteria. Biochemical tests were used to identify the isolates which retained antimicrobial activity as determined by spot assay or that were isolated as representatives of the turbot-rearing microbiota. Gram stain (Bactident aminopeptidase; Merck, Darmstadt, Germany), catalase (3% H_2O_2), and oxidase (BBL Oxidase Dryslide; BD) reactions were tested on cultures grown for 1 day on MA at 25°C. Shape, motility, and ability to form rosettes were examined by phase-contrast microscopy of cultures grown in MB for 3 days at 25°C under stagnant conditions. The ability to ferment or oxidize glucose was tested in OF basal medium (Merck) (30) supplemented with 2% Instant Ocean. Fermentative strains were grown for 1 day at 25°C in 5 ml MB and streaked on plates selective for vibrios (TCBS cholera medium [Oxoid CM333]), and the cultures were also tested for sensitivity to vibriostaticum (2,4-diamino-6,7-di-isopropylteridine; 0/129 DD0014, 10 μg, and DD0015, 150 μg; Oxoid) on MA. All plates were incubated at 25°C for 24 h.

Bioinformatics on *Roseobacter* clade strains. The 16S rRNA genes of presumed *Roseobacter* clade strains (gram-negative, nonfermentative/nonoxidative, motile rods with positive catalase and oxidase reactions and the ability to form rosettes and brownish pigment) were sequenced not only to identify the isolates but also to determine similarities of the strains isolated from the Danish and Spanish turbot-rearing units. The gyrase B (*gyrB*) genes were sequenced to determine if the very homogenous clusters found by comparing 16S rRNA genes were also reflected in this housekeeping gene.

DNA was purified from cultures grown for 3 days at 25°C in 5 ml MB using the Dynal Dynabeads DNA Direct System (Dynal Biotech ASA, Oslo, Norway). Two microliters DNA was mixed with 13 μl 2× Brilliant IIQPCR Master Mix (Stratagene, La Jolla, CA), 8 μl sterile MilliQ water, and 1 μl 12.5 M of each primer. The primers were synthesized by DNA Technology A/S (Aarhus, Denmark), and we used 27F (5′ AGAGTTTGATCMTGGCTCAG 3′) and 1492R (5′ TACGG YTACCTTGTTACGACTT 3′) for 16S rRNA genes and UP-1 (5′ GAAGTCA TCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA 3′) and UP-2 (5′ AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT 3′) for *gyrB* genes (52). The PCRs for 16S rRNA genes were run (9800 Fast Thermal Cycler; Applied Biosystems, Foster City, CA) for 10 min at 95°C before 35 cycles of 95°C for 30 s, 51°C for 1 min, and 72°C for 1.5 min and, after the last cycle, 7 min at 72°C. For the *gyrB* genes, the reactions were run for 10 min at 95°C followed by 40 cycles of 1 min at 95°C, 1 min at 60°C, and 2 min at 72°C. The program ended with 7 min at 72°C. All PCR products were analyzed by 1% agarose gel electrophoresis, bands were cut out, and DNA was purified (GFX PCR DNA and gel band purification kit; GE Healthcare, Buckinghamshire, Great Britain). Sequencing was done by DNA Technology A/S using the primer set 518F (sequence, 5′ CCAGCAGCCGCGGTAATACG 3′) and 800R (sequence, 5′ TACCAGGGTATCTAATCC 3′) for 16S rRNA genes and UP1S (5′ GAAGTCATCATGACCGTTCTGCA 3′) and UP2Sr (5′ AGCAGGGTACGG ATGTGCGAGCC 3′) for *gyrB* genes (52).

Sequences were assembled using Vector NTI (Invitrogen, Carlsbad, CA). BLASTN searches using 16S rRNA sequences were performed on the NCBI database to find sequences with ≥98% identity. Relevant type strains for species identified were found online in the List of Prokaryotic Names with Standing in Nomenclature (15) and included in the analysis. Multiple alignments were done using the program ClustalX (48), and the alignments were edited in BioEdit (25). Distance matrix JC was calculated using ClustalX and neighbor joining (45), and bootstrap (number of trials, 100) (18) trees were drawn using the program MEGA4 (47). Type strains *Rhodobacter capsulatus* ATCC 11166 plus *Rhodobacter sphaeroides* ATCC 17023 and *Roseobacter denitrificans* Och114 plus *Roseobacter litoralis* Och149 served as outgroups in the 16S rRNA and *gyrB* trees, respectively.

Subtyping of *Roseobacter* clade strains by RAPD. *Roseobacter* clade isolates were random amplified polymorphic DNA (RAPD) typed as described earlier (50) to determine subspecies homology. In brief, 2 μl purified DNA plus one Ready-To-Go RAPD Analysis Bead (Amersham Pharmacia Biotech Inc., Piscataway, NJ) was dissolved in 23 μl of one of the following 1 μM primers (DNA Technology A/S): UBC 104 (sequence 5′ GGGCAATGAT 3′) and UBC 106 (sequence 5′ CGTCTGCCCG 3′) (29). The PCRs were run at 95°C for 2 min followed by 10 cycles of 1 min at 94°C, annealing at 45°C for 1 min, and extension at 72°C for 2 min. The annealing temperature was decreased by 1°C per cycle. Then 30 cycles followed with denaturing at 94°C for 1 min, annealing at 35°C for 1 min, and extension at 72°C for 2 min. The program was completed with 10 min of final extension at 72°C. The bands were visualized after electrophoresis in 2% agarose gels by staining with ethidium bromide. *Phaeobacter*

strains (27-4, 8-1, and 632-1) and a 100-bp ladder (Amersham) standard were included in all gels.

Influence of growth conditions on attachment, pigment formation, and antibacterial activity of *Roseobacter* clade strains. All *Roseobacter* clade strains were grown in 5 ml MB for 3 days at 25°C under stagnant conditions, and 200 μl was reinoculated in 20 ml MB in 250-ml bottles and grown for 3 days at 25°C under stagnant or shaking (200 rpm) conditions. Sterile-filtered supernatants were used for testing antimicrobial activity in a well diffusion assay in Instant Ocean agar as described above and for measuring pigment by spectroscopy (Novaspec II; Pharmacia Biotech, Cambridge, England) at 398 nm (9). Cell numbers were determined on cultures by 10-fold dilutions in sterile 0.85% saline and plated on MA, and plates were incubated at 25°C for 3 days. A subset of seven *Roseobacter* clade strains from the Danish turbot larval farm, four from the Spanish turbot larval farm, the strain from a dinoflagellate culture, and the *V. anguillarum* strain not able to form rosettes were examined for their ability to attach to surfaces as described by Bruhn et al. (8). Briefly, glass coverslips (Knittel Glässer, Braunschweig, Germany) were dipped for 5 s in cultures grown in 20 ml MB in 250-ml bottles for 3 days at 25°C under stagnant or shaking (200 rpm) conditions as described above. Loosely attached cells were removed by placing the coverslip on absorbent paper, after which the remaining cells were fixed at 60°C for 30 min. Attached cells were stained for 15 min in 0.1% crystal violet, and unbound dye was washed off using phosphate-buffered saline (BR0014G; Oxoid). Dye bound to attached cells was dissolved in 2 ml 33% acetic acid, and the optical density at 590 nm (OD_{590}) was measured. The attachment was done in duplicate. The crystal violet OD resulting from dipping glass coverslips in pure MB were subtracted from all measurements. Phase-contrast microscopy was performed on cells from the air-medium interface of the cultures. Pigment was measured as described earlier (9). The antibacterial compound TDA was measured in sterile-filtered supernatant as described below.

HPLC-tandem mass spectrometry analysis of TDA. Extracts of 5 μl were analyzed on an Agilent (Torrance, CA) 1100 high-pressure liquid chromatography (HPLC) system controlled by MassLynx V4.1. Samples were separated on a Gemini C_6-phenyl 3-μm, 2-mm-inside-diameter × 50-mm column (Phenomenex, Torrance, CA), using a flow rate of 0.300 ml/min at 25°C. A linear water-acetonitrile (ACN) gradient was used, starting at 10% ACN, going to 45% ACN in 8 min and then 100% ACN in 0.5 min, holding this for 2 min before reverting to 10% ACN in 1 min, and maintaining this for 8 min. Both solvents contained 120 mM formic acid. The HPLC was a coupled Quattro Ultima triple mass spectrometer (Waters-Micromass, Manchester, United Kingdom) with a Z-spray electrospray ionization source using a flow rate of 700 liters/h nitrogen at 350°C; hexapole 1 was held at 30 V, and the cone was held at 25 V. Nitrogen was used as collision gas, and the mass spectrometer operated in positive multiple-reaction monitoring mode (dwell time, 100 ms), monitoring *m/z* 213 to 151 (25-V collision energy) and *m/z* 213 to 167 (20-V collision energy) as quantifier and qualifier ions, respectively.

Nucleotide sequence accession numbers. The 16S rRNA and *gyrB* gene sequences have been deposited in GenBank under the accession numbers FJ014969 to FJ01503 and FJ014947 to FJ014968, respectively.

TABLE 1. Numbers of samples taken at a Danish turbot larval farm and numbers of isolates with antagonistic activity against *Vibrio anguillarum* strain 90-11-287 in seawater agar

Sample site	No. of samples		No. of antagonistic isolates		
	Total	No. with antagonistic bacteria determined by replica plating	Total	Determined by spot assay	Determined by well diffusion assay
Inside production					
Fish tanks					
Water	4	3	12	11	5
Surface	9	3	7	7	6
Water cleaning system					
Water	3	3	13	12	5
Surface	2	2	10	9	9
Tank with copepod cultures					
Suspension	4	3	9	2	0
Surface	4	4	11	8	0
Bag with algal culture, suspension	4	3	11	11	9
Subtotal	30	21 (70%)	73	60 (82%)	34 (47%)
Outside production					
Tanks with larvae					
Water	2	2	11	11	6
Surface	2	1	1	1	1
Tanks with zooplankton					
Water	2	2	11	9	5
Surface	2	1	7	6	6
Water cleaning system, surface	1	1	2	2	0
Tank with water from the inlet					
Water	1	1	5	5	0
Surface	1	1	3	2	0
Tank with nauplii					
Water	1	1	4	4	2
Surface	1	0	0	0	0
Subtotal	13	31 (76%)	44	40 (90%)	20 (45%)
Grand total	43	31 (76%)	117	100 (85%)	54 (46%)

RESULTS

Sampling, isolation, and identification of antimicrobial strains. Forty-three samples from the Danish turbot larval farm (Table 1) all had aerobic plate counts of 10^5 to 10^6 CFU/ml on MA. Thirty-one samples contained colonies that in the replica assay indicated inhibition of *V. anguillarum*. We isolated cells from 117 of these colonies, and upon retesting, the inhibitory activity was retained for 100 isolates. Fifty-four of the 100 isolates also inhibited *V. anguillarum* 90-11-287 when we used sterile-filtered supernatant from stagnant cultures in the well diffusion assay. These isolates originated from water (23 isolates), tank surfaces (22 isolates), and algal cultures (nine isolates). Instant Ocean agar was used for all subsequent experiments, as this is easier to standardize than an agar based on natural seawater, and gave very similar inhibitory profiles. Fifty-one of the 54 isolates were gram-negative, nonfermentative/nonoxidative, motile rods with positive catalase and oxidase reactions and rosette formation and therefore were likely to belong to the *Roseobacter* clade. It was not possible, based on the biochemical tests used, to identify the last three isolates. The 46 isolates that were antagonistic only in the spot assay and not in the well diffusion assay were primarily identified as *Vibrio* spp. (38 out of 46), being fermentative, gram-negative rods able to grow on TCBS agar and in the presence of vibriostaticum.

BLASTN searches supported the *Roseobacter* clade affiliation of the 51 isolates, as all 16S rRNA gene sequences showed ≥98% similarity to species such as *Roseobacter* spp., *P. inhibens*, *P. gallaeciensis*, and *Phaeobacter daeponensis*, whereas isolates from algal cultures were ≥98% similar to *Ruegeria mobilis*, *Ruegeria pelagia*, and *Silicibacter* spp. (Table 2).

RAPD subtyping of *Roseobacter* clade strains. RAPD typing divided the 51 *Roseobacter* clade strains into 14 different subtypes when two primers were used (data not shown). None of these types were similar to three major RAPD types that had been found in the Spanish turbot-rearing farms (29). Three major subgroups found in the Danish turbot-rearing unit were designated AA, BB, and CC and harbored 23, 4, and 9 strains, respectively. All the 23 subtype AA strains originated from

TABLE 2. Identification of antagonistic bacterial strains isolated from a Danish turbot larval farm

Sampling site	No. of isolates antagonistic in:					
	Both spot and well diffusion assays[a]			Spot but not well diffusion assay[b]		
	Phaeobacter spp.	*Ruegeria* spp.	Others	*Roseobacter*-like	*Vibrio* spp.	Others
Indoor production						
Fish tanks						
Water	5	0	0	0	6	0
Surface	6	0	0	0	1	0
Water cleaning system						
Water	5	0	0	0	7	0
Surface	9	0	0	0	0	0
Tank with copepod cultures						
Suspension	0	0	0	0	1	1
Surface	0	0	0	0	4	4
Bag with algal culture, suspension	0	9	0	1	0	1
Subtotal	25	9	0	1	19	6
Outdoor production						
Tanks with larvae						
Water	6	0	0	0	5	0
Surface	1	0	0	0	2	0
Tanks with zooplankton						
Water	4	0	1	0	4	0
Surface	6	0	0	0	0	0
Water cleaning system, surface	0	0	0	0	2	0
Tank with water from the inlet						
Water	0	0	0	0	5	0
Surface	0	0	0	0	0	0
Tank with nauplii						
Water	0	0	2	0	1	1
Surface	0	0	0	0	0	0
Subtotal	17	0	3	0	19	1
Grand total	42	9	3	1	38	7

[a] Identified by BLASTN search with 16S rRNA gene sequences on the NCBI database.
[b] Identified by biochemical tests (Gram stain, catalase and oxidase tests, ability to metabolize glucose, reaction on TCBS plates, and sensitivity/resistance to the vibriostatic agent 0129) and microscopy (motility, shape, and ability to form rosettes).

parts of the production site that were indoors. Only two indoor isolates belonged to other subtypes (BB and DD). The RAPD type CC encompassed nine strains, and all were isolated from the algal cultures. All of these nine strains were subsequently identified by 16S rRNA gene sequencing as *Ruegeria* spp. Subtype BB harbored four strains isolated at different sites. The remaining 17 isolates were isolated from the outdoor sites of the production unit and made up a total of 11 RAPD types.

16S rRNA and *gyrB* gene comparison of *Roseobacter* clade strains. Three major clusters appeared when comparing 989 bases of the 16S rRNA gene from 48 Danish and nine Spanish *Roseobacter* clade isolates (Fig. 1a). Three Danish isolates could not be amplified. One cluster was identical to type strains *R. mobilis* MBIC01146 and *R. pelagia* HTCC2662 and contained nine isolates representing two samples from Danish algal cultures and one Spanish isolate. Twenty-one Danish isolates from seven indoor samples (all RAPD type AA) and eight isolates from the Spanish turbot farm clustered together with the type strain *P. gallaeciensis* BS107. The last cluster consisted of the type strain *P. inhibens* T5 and Danish isolates of different RAPD types. Fifteen isolates were from five outdoor samples, and two isolates were taken from two different indoor samples. The *gyrB* gene (998 bases) was sequenced for 19 Danish and three Spanish strains representing the different clusters obtained in the 16S rRNA tree. It resulted in the same three major clusters when this housekeeping gene was used as did the 16S rRNA gene sequences; however, a greater separation between the Danish and the Spanish strains was achieved using the *gyrB* sequences (Fig. 1b). Furthermore, the Spanish isolate 8-1 was affiliated with the *P. gallaeciensis* BS107 cluster in the 16S rRNA tree whereas in the *gyrB* tree it grouped with the strains from the *P. inhibens* T5 cluster.

The *gyrB* sequences of some isolates revealed putative stop codons in five isolates (*Phaeobacter* strains M3-1.2, M4-3.1, M6-4.1, M9-4.2, and M9-4.3) or frameshift mutations in 12 isolates (*Phaeobacter* strains 8-1, 27-4, M2-3.1, M2-4.2, M3-1.3, M4-3.1, M6-4.1, M9-4.1, M9-4.2, and M9-4.3 and *Ruegeria* strains M41-3.1 and M43-1); however, the reason for this or the functional implications have not been further pursued.

Inhibition of the general microbiota by *Roseobacter* clade strains. Seventeen isolates randomly collected from water and

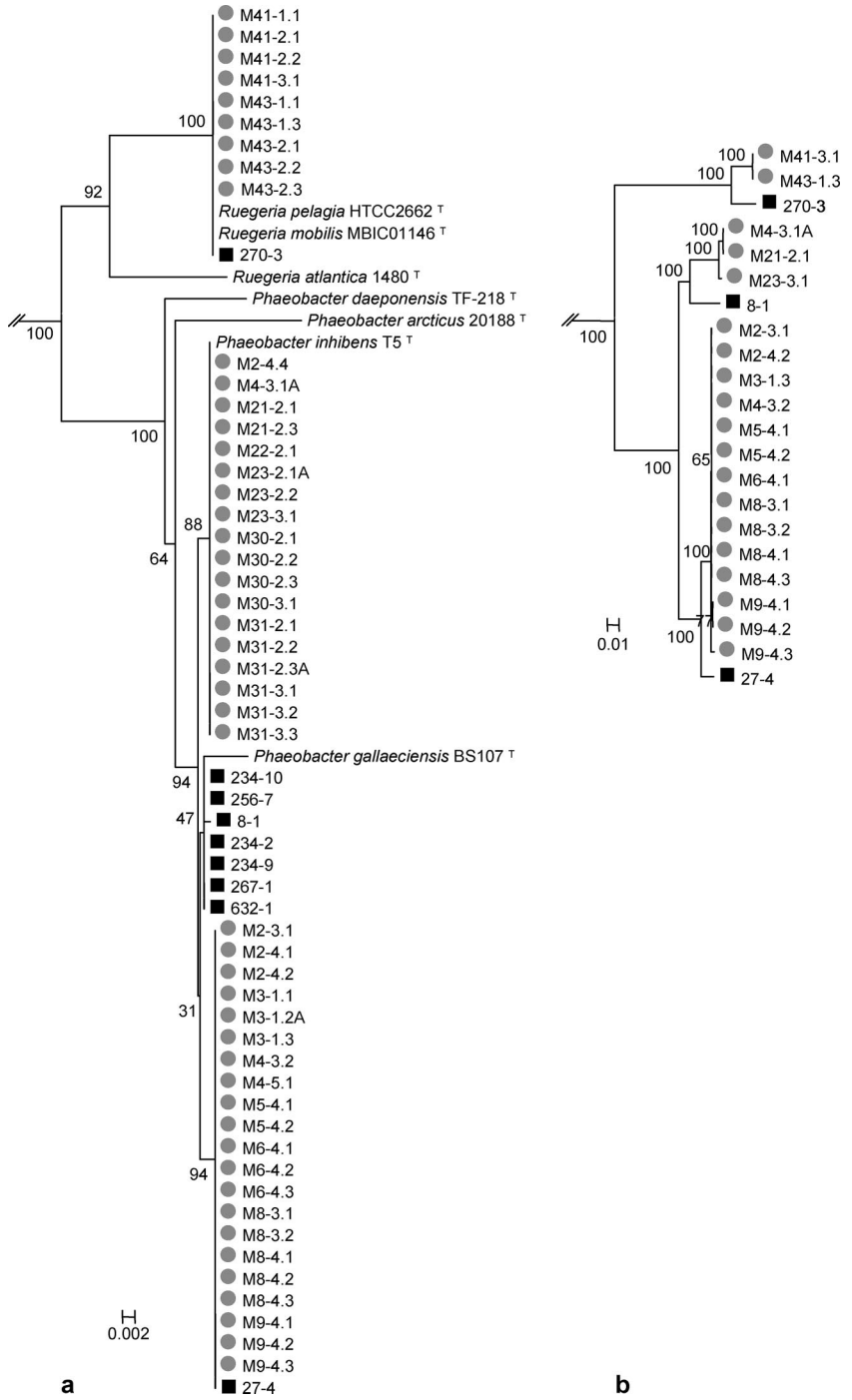

FIG. 1. Phylogenetic trees constructed using the 16S rRNA gene (a) and gyrase B (*gyrB*) gene (b) sequences. Numbers at the nodes are bootstrap values from 100 replicates. Type strains *Rhodobacter capsulatus* ATCC 11166 and *Rhodobacter sphaeroides* ATCC 17023 served as outgroups in the 16S rRNA tree, and *Roseobacter denitrificans* Och114 and *Roseobacter litoralis* Och149 served as outgroups in the *gyrB* gene trees (not shown). ● and ■, Danish and Spanish turbot-rearing farm strains, respectively. T, type strains.

surface samples from the Danish turbot larval farm were inhibited by the 51 *Roseobacter* clade strains, although some of the strains were inhibited to a lesser degree than was *V. anguillarum* 90-11-287, as indicated by the size of the inhibition zone (Table 3). The inhibition zones surrounding the 42 *Phaeobacter* sp. strains in the spot assay were larger than the zones produced by the nine *Ruegeria* sp. strains. This pattern was especially pronounced for target strains isolated from water samples. The 17 isolates were identified as eight different genera or species, and one isolate was impossible to identify based on the methods used. The *Phaeobacter* spp. strongly inhibited all but one isolate (*Rhodococcus* sp.), whereas the

TABLE 3. Abilities of *Phaeobacter* spp. and *Ruegeria* spp. to inhibit 17 isolates from a Danish turbot larval farm[e]

Identification of isolates from sample[a]	Grading of the size of inhibition zone in spot assay for[b]:	
	Phaeobacter spp. (42 strains)	*Ruegeria* spp. (9 strains)
Water, indoor fish tank		
Halomonas sp./*Cobetia marina*[c]	3.0 ± 0.0	1.0 ± 0.0
Halomonas sp./*Cobetia marina*	3.0 ± 0.0	1.6 ± 0.5
Micrococcus sp.	3.0 ± 0.2	2.9 ± 0.3
Halomonas sp./*Cobetia marina*	3.0 ± 0.0	0.7 ± 0.5
Water, outdoor tank with phytoplankton and zooplankton		
Pseudomonas sp.	3.0 ± 0.0	0.9 ± 0.8
Pseudomonas sp.	3.0 ± 0.2	0.4 ± 0.9
Pseudoalteromonas sp.	2.0 ± 0.5	0.0 ± 0.0
Water, indoor fish tank		
Pseudoalteromonas sp.	3.0 ± 0.0	2.0 ± 0.9
Micrococcus sp.	2.9 ± 0.3	2.4 ± 0.5
Marinomonas sp.	3.0 ± 0.0	2.3 ± 1.0
Surface, indoor fish tank		
Rhodococcus sp.	1.0 ± 0.0	0.0 ± 0.0
Surface, outdoor tank with phytoplankton and zooplankton		
Micrococcus sp.	3.0 ± 0.0	3.0 ± 0.0
Surface, indoor fish tank		
Olleya marilimosa	2.9 ± 0.3	2.1 ± 0.9
Unidentified[d]	2.6 ± 0.5	3.0 ± 0.0
Kordia algicida	3.0 ± 0.2	2.0 ± 0.5
Pseudoalteromonas sp.	2.8 ± 0.7	2.4 ± 0.7
Micrococcus sp.	2.7 ± 0.7	2.1 ± 0.9
Diseased rainbow trout (*Oncorhyncus mykiss*)		
Vibrio anguillarum 90-11-287	3.0 ± 0.3	2.7 ± 0.7

[a] Isolates were identified by biochemical tests (Gram stain, catalase and oxidase tests, ability to metabolize glucose, reaction on TCBS plates, sensitivity/resistance to vibriostatic agent 0129), microscopy (motility and shape), and BLASTN searching of 16S rRNA gene sequences in the NCBI database.

[b] Grades (0 to 3) indicate the size of the clearing zone around the spot of colony mass: 0, no zone; 1, <0.5-mm zone; 2, 0.5- to 2-mm zone; 3, >2-mm zone. Values are averages and standard deviations of 42 *Phaeobacter* sp. and nine *Ruegeria* sp. strains, respectively.

[c] *Cobetia marina* (reclassified from *Halomonas marina* [3]) and *Halomonas* sp. both belong to the *Halomonadaceae* family.

[d] Not possible to identify based on the biochemical tests, and BLASTN search gave low identities to other known species (≤96%).

[e] Target bacteria were seeded in agar, and *Phaeobacter* spp. or *Ruegeria* spp. were added on top in a spot assay.

Ruegeria spp. inhibited all but six isolates (*Halomonas* sp./*Cobetia marina*, *Pseudomonas* sp., *Pseudoalteromonas* sp., and *Rhodococcus* sp.). There was no pattern in the sensitivity of the target strains to *Roseobacter* clade strains depending on genus or species of the target organism.

Influence of growth conditions on attachment, pigment formation, and antibacterial activity of *Roseobacter* clade strains. All the 51 Danish and nine Spanish *Roseobacter* clade strains produced brown pigment (as determined by OD_{398}) when grown under stagnant conditions in MB. However, 42 Danish and seven Spanish isolates also produced pigment when grown under aerated (shaking) conditions, although the amount of pigment was less than that in stagnant cultures (data not shown), and all these strains were identified as *Phaeobacter* spp. The brown pigment correlated with the antibacterial activity of the supernatant in the well diffusion assay, as inhibition was observed only when pigment was produced. The remaining nine Danish and two Spanish isolates expressed these phenotypes only during stagnant growth. Of these, all Danish strains and one Spanish strain were identified as *Ruegeria* spp., and the last Spanish strain behaving like the *Ruegeria* spp. was *Phaeobacter* strain 27-4. All stagnant and shaken cultures grew to approximately 5×10^8 CFU/ml and 1×10^9 CFU/ml, respectively. It was examined for a subset of samples (eight *Phaeobacter* sp. strains, three *Ruegeria* sp. strains, *Silicibacter* strain TM1040, and *V. anguillarum* 90-11-287) if rosette formation and attachment ability co-occurred with pigment production and antibacterial activity as seen for *Phaeobacter* strain 27-4 (8, 9). All *Roseobacter* clade isolates attached better to the glass surface as determined by crystal violet staining when grown under stagnant conditions (Fig. 2), and more cells appeared in a rosette morphology under stagnant conditions than under shaking conditions (Fig. 3) (data are shown only for the Danish *Phaeobacter* strain M23-3.1, the Spanish *Phaeobacter* strain 27-4, and the Danish *Ruegeria* strain M43-2.3 but are typical for the other *Roseobacter* clade strains). This was also the case for *Phaeobacter* spp. and *Silicibacter* strain TM1040, which produced brown pigment and had antibacterial activity when grown under shaking conditions. For all tested strains, production of TDA correlated with pigment formation and antibacterial activity (Fig. 2).

DISCUSSION

Roseobacter clade strains capable of in vitro inhibition of the fish-pathogenic bacterium *V. anguillarum* were found in many sites of a Danish turbot larval farm. In fact, these bacteria strongly dominated the antagonistic culturable microbiota (51 out of 54 isolates). *Roseobacter* spp. have been isolated from a Spanish turbot larval unit (29) and from scallop larval cultures (43), and Brunvold et al. (10) detected a band using denaturing gradient gel electrophoresis with sequence homology to a *Roseobacter* sp. strain from a cod hatchery. This, collectively, suggests that *Roseobacter* clade members are common in this type of environment. We did, like Hjelm et al. (29), also identify antagonistic *Vibrio* spp. from the turbot larval rearing unit; however, these were not further characterized, as they are likely pathogenic for the turbot (28).

Roseobacter clade strains are often associated with surface colonization in the marine environment (12, 13) and were isolated predominantly from surfaces in a Spanish turbot farm (29). However, in the present study of the Danish turbot farm, they were equally prevalent in the tank water, and they have indeed also been isolated as pelagic bacteria in marine environments (11, 40). *Roseobacter* clade strains are often associated with algal blooms in marine environments and can account for as much as 50% of the bacterial rRNA genes (1, 11, 22). In concordance with this niche preference, we isolated several strains from the algal cultures. This particular association may render the algae an interesting vector for supplying the probiotic culture to the fish larval rearing process.

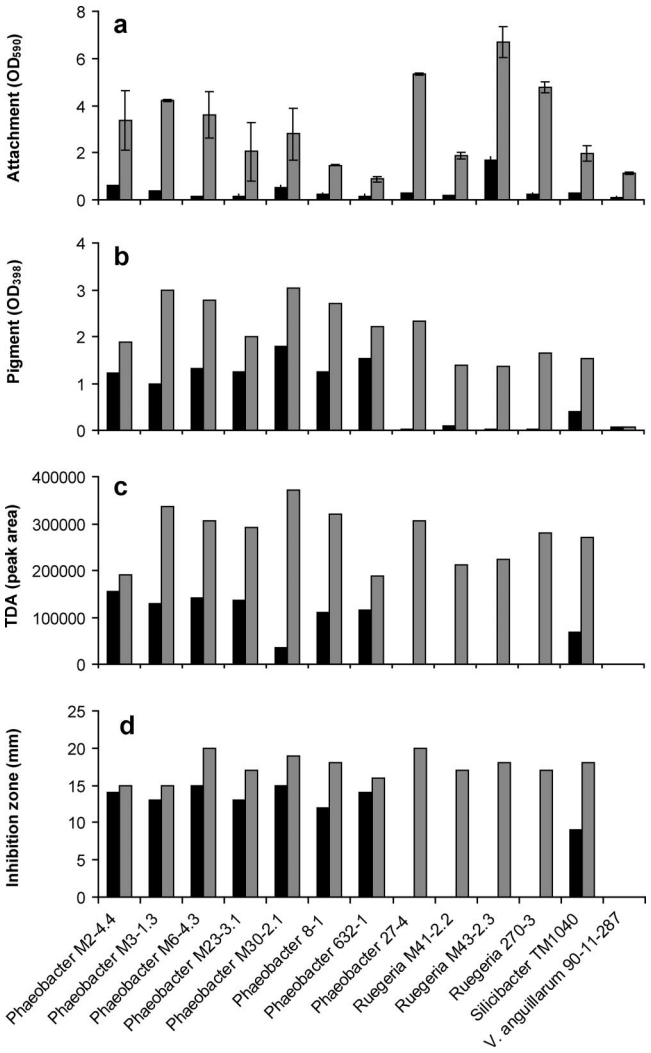

FIG. 2. Attachment (a), production of pigment (b), production of TDA (c), and ability to inhibit *Vibrio anguillarum* 90-11-287 in well diffusion assay (d) of *Roseobacter* clade strains and *Vibrio anguillarum* 90-11-287 grown under shaking (200 rpm) (black bars) or stagnant (0 rpm) (gray bars) conditions. The attachment experiment was conducted in duplicate, and error bars represent 1 standard deviation. When measuring TDA, the HPLC-tandem mass spectrometry peak area from the *m/z* 213 to 151 transition was used. In the well diffusion assay, the diameter of the well itself has been subtracted from the diameter of the inhibition zone.

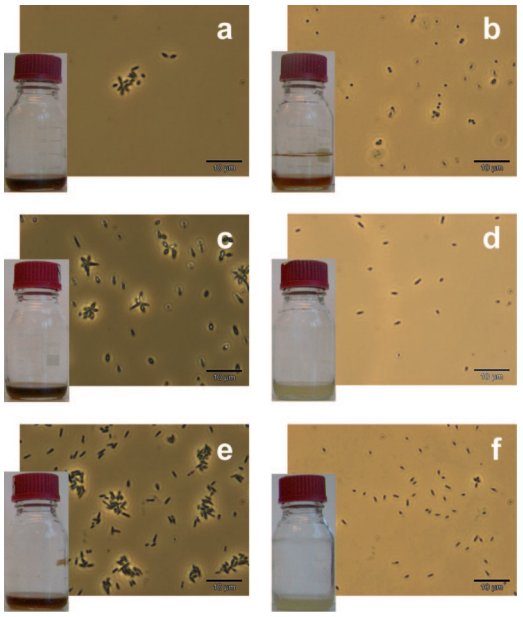

FIG. 3. Cell morphology of and pigment formation by *Roseobacter* clade strains grown in MB under static (0 rpm) (a, c, and e) or shaking (200 rpm) (b, d, and f) conditions. The Danish *Phaeobacter* sp. strain M23-3.1 (a and b), the Spanish *Phaeobacter* sp. strain 27-4 (c and d), and the Danish *Ruegeria* sp. strain M43-2.3 (e and f) are shown. Microscopy pictures are from phase-contrast microscopy at ×1,000 magnification. Bars, 10 μm.

Comparing 989 bases of the 16S rRNA gene revealed three clusters among the Danish and Spanish turbot-rearing farm isolates. Strains similar to *P. gallaeciensis* BS107 (type strain) dominated among the indoor isolates, whereas outdoor strains were similar to *P. inhibens* T5 (type strain), and all isolates from algal cultures were similar to type strains *R. mobilis* MBIC01146 and *R. pelagia* HTCC2662. The two latter type strains are identical on the 16S rRNA level. This indicates that different species of the *Roseobacter* clade colonize specific niches in the Danish rearing unit. All four species from the *Roseobacter* clade are very common in marine environments (11), and as they are easily culturable (6, 32, 38, 43), it is not surprising to find them in a nutrient-rich environment favoring fast-growing heterotrophic organisms. The 16S rRNA gene is very useful as a phylogenetic comparative gene for a number of genera and species; however, it may not be the optimal differentiating molecule in some genera (14, 49). We considered that the *Roseobacter* clade species could, in principle, be difficult to differentiate based on 16S rRNA genes and therefore chose to compare the strains using the *gyrB* gene also, which has been used successfully to discriminate between genera and species with closely related 16S rRNA gene sequences (19, 26, 42, 53). In this study, the *gyrB* gene supported the finding of the clusters obtained with 16S rRNA gene sequences, but at the same time it gave a greater evolutionary distance between the Danish and Spanish strains. To our knowledge, this is the first time that the *gyrB* gene has been used for phylogenetic analysis of *Roseobacter* clade members.

Subtyping the Danish *Roseobacter* clade strains using RAPD with two primers revealed types other than those found in the Spanish turbot larval farm (29). The two turbot farms have different water sources (Limfjorden, a fiord in Denmark, versus the Atlantic Ocean off the Galician coast). Also, specific subtypes may have preferences for special niches or may have been introduced at random and remained. This is indicated by the fact that even though the same water source was used in the Danish turbot larval farm for inside and outside production, only one out of 14 RAPD types was found at both sites. The diversity in terms of subtypes was much lower in the indoor production than in the outside production sites. A similar phenomenon has been reported for the human-pathogenic bacterium *Listeria monocytogenes* (27), which is an environmental bacterium capable of colonizing fish-processing units. This could be because the outdoor production is an open

production form and thereby more easily influenced by the surrounding environment than is the indoor production.

The *Phaeobacter* spp. and *Ruegeria* spp. isolated due to their antimicrobial activity against *V. anguillarum* also inhibited bacterial strains randomly isolated from surfaces and water from fish tanks in the turbot farm. This is in agreement with the other studies, as alphaproteobacteria can inhibit different marine bacteria (24, 33), and *Phaeobacter* spp. inhibit organisms as diverse as flavobacteria, *Acinetobacter*, vibrios, *Pseodoalteromonas*, *Alteromonas*, *Bacillus*, and *Halomonas* (5, 6, 8, 9, 28, 41). Hence, one should be aware that if such bacteria are deliberately added as probiotic organisms, they may not just inhibit the pathogenic agents but also alter the general microbiota. Therefore, careful supervision of the changes in microbiota upon probiotic additions is required.

Phaeobacter spp. appeared more inhibitory than *Ruegeria* spp.; however, the two species produced TDA in similar amounts when grown in stagnant broth cultures (Fig. 2c). We therefore speculate that *Phaeobacter* spp. may produce inhibitory compounds other than TDA and that these act as antimicrobials themselves or act synergistically with TDA. The antagonistic activity of *Phaeobacter* spp. and *Ruegeria* spp. is likely to be an important factor explaining their dominance in several niches, and indeed they outcompete other marine organisms such as *Pseudoalteromonas tunicata* in competition experiments (41).

The ability of all the *Roseobacter* clade strains tested to inhibit *V. anguillarum* co-occurred with their production of brown pigment and was independent of the origin of the isolates. A similar coupling has been reported for *P. inhibens* T5, *Phaeobacter* strain 27-4, and *Silicibacter* strain TM1040 (6, 8, 9). While the inhibitory compound TDA is not the pigment itself, the coupling between these two phenotypes has also been demonstrated by transposon mutagenesis creating noninhibitory mutants in a *Phaeobacter* strain and a *Silicibacter* strain (20). The production of pigment and antibacterial activity was influenced by growth conditions; however, this varied with species, as the *Ruegeria* spp. behaved like *Phaeobacter* strain 27-4 (9), producing pigment and showing antimicrobial activity only when grown under stagnant conditions. In contrast, *Phaeobacter* spp. from the Danish turbot larval farm expressed these phenotypes after both stagnant and aerated growth. This is interesting from an application point of view, as only a few sites in a fish tank will be stagnant, and hence, the *Phaeobacter* spp. isolated in the present study may hold greater promise as probiotic organisms than *Phaeobacter* strain 27-4. It must be determined if the Danish *Phaeobacter* strains are capable of disease suppression in vivo as has been documented for strain 27-4 (39). As seen in this study, strains with 100% similarity in the 16S rRNA gene sequences do not necessarily express phenotypes in the same pattern. The same phenomenon was observed by Grossart et al. (24), as isolates showed different inhibitory activities against marine bacteria. Also, *Silicibacter* strains (*Silicibacter* strain TM1040 and *Silicibacter pomeroyi* strain DSS-3) vary in how the culture conditions influence pigment production and antimicrobial activity (8). In the present study, the production of TDA, pigment, and antimicrobial activity co-occurred for a subset of 12 *Roseobacter* clade strains (eight of *Phaeobacter* spp., three of *Ruegeria* spp., and one of *Silicibacter* sp.). TDA has been detected from *Phaeobacter* spp. and *Silicibacter* strain TM1040 (6, 9, 20), and to the best of our knowledge the present study is the first report of TDA production by *R. mobilis*/*R. pelagia* strains.

A prominent characteristic of *Phaeobacter* and some other *Roseobacter* clade strains is their ability to grow as rosettes (8, 9), and this mode of growth appears to enhance the surface attachment ability of organisms. Also, the strains isolated in the present study grew as rosettes, and this did correlate, for *Ruegeria*, with their attachment capability.

In conclusion, *Phaeobacter* spp. and *Ruegeria* spp. with antibacterial activity colonized different units in the Danish turbot larval farm. We suggest that members of the *Roseobacter* clade are common colonizers of marine larval rearing units. This makes the clade a suitable candidate as a universal marine fish larval probiotic bacterium. In particular the Danish strains of *Phaeobacter* spp. are of applied interest as their antibacterial activity and TDA production occurred under several types of growth conditions. However, the true probiotic potential will have to be further evaluated in in vitro and challenge trials.

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Paper 2

Cisse Hedegaard Porsby, Mark A. Webber, Kristian Fog Nielsen, Laura J. V. Piddock and Lone Gram

Resistance and tolerance to tropodithietic acid, an antimicrobial in aquaculture, is hard to select

Antimicrobial Agents and Chemotherapy: ***Submitted***

Resistance and Tolerance to Tropodithietic Acid, an Antimicrobial in Aquaculture, is Hard to Select

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Running title: Lack of resistance and tolerance to TDA

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ABSTRACT

The antibacterial compound tropodithietic acid (TDA) is produced by bacteria of the marine *Roseobacter* clade and is thought to explain the fish probiotic properties of some roseobacters. The aim of the present study was to determine the antibacterial spectrum of TDA and the likelihood of resistance development. TDA was effective against a range of human pathogenic bacteria including strains resistant to conventional antibiotics. The MICs of TDA were higher for Gram negative bacteria than Gram positive bacteria. TDA was bactericidal against *Salmonella enterica* serovar Typhimurium SL1344 and *Staphylococcus aureus* NCTC 12493 and killed both growing and non-growing cells. Several experimental approaches were used to select mutants resistant to TDA or sub-populations of strains with enhanced tolerance to TDA. No approach (single exposures to TDA administered via different methods, screening of a Tn-library for resistant mutants or prolonged exposure to incremental concentrations of TDA) resulted in resistant or tolerant strains. After more than 300 generations exposed to sub-MIC and MIC concentrations of TDA, strains tolerant to 2xMIC of TDA for wildtype strains were selected but the tolerance disappeared after one passage in medium without TDA. *S.* Typhimurium mutants with non-functional efflux pump and porin genes had the same TDA susceptibility as wildtype strains suggesting that efflux pumps and porins are not involved in innate tolerance to TDA. TDA is a promising broad spectrum antimicrobial in part due to the fact that enhanced tolerance is difficult to gain and that the TDA tolerant phenotype appears to only confer low level resistance and is very unstable.

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INTRODUCTION

The *Roseobacter* clade, which belongs to the α-proteobacteria, constitute a major part of oceanic bacterioplankton communities (6,17,46) and many *Roseobacter* clade strains are easily culturable (3,20,41). The success of the *Roseobacter* clade bacteria may be explained by several factors one of which is their production of secondary metabolites that may give them a competitive advantage over other species (19,28,31). This includes the antibacterial compound tropodithietic acid (TDA) which is produced by several *Roseobacter* species such as *Phaeobacter gallaeciensis*, *Phaeobacter inhibens* and *Ruegeria mobilis* (3,5,41).

Ph. gallaciencis and *R. mobilis* are of great interest in the aquaculture sector for use as probiotics as they kill or inhibit growth of several fish pathogenic bacteria and they improve survival of fish larvae in challenge trials (14,22,40). The killing or inhibitory effect appears related to TDA production (3,22,42). In fact, D'Alvise et al. (2010) showed that the fish pathogenic bacterium *Vibrio anguillarum* strain 90-11-287 was only killed by a TDA producing wild type of *Phaeobacter* strain 27-4 and not by a mutant deficient in TDA production (13).

The ability of TDA to inhibit several bacteria that are pathogenic to fish and the possible use of TDA producing bacteria as probiotics in aquaculture raises a number of issues and questions, and some are addressed in the present study. TDA has antibacterial action against bacteria outside of the marine or aquatic environment including human pathogenic bacteria (3,27,49) and could also be of interest as an antibacterial outside the aquaculture sector. However, a wide spread use of TDA could be potentially detrimental if resistance and subsequent cross-resistance to other agents develops, as it has been the case for the majority of drugs discovered so far (11).

TDA is a disulfide tropolone (8) and it exists in a tautomeric form with thiotropocin (21). Thiotropocin (or tropodithietic acid) was first isolated from a soil bacterium identified as a *Pseudomonas* (8,27) and in addition to *Roseobacter*-clade bacteria, the compound has also been isolated from marine *Caulobacter* spp. and *Pseudovibrio* strains (15,25). It is antibacterial against several bacteria including *Escherichia coli* and *Pseudomonas aeruginosa* (3,27,49) and is also inhibitory against some mammalian cells (26). Tropolones with other side groups than a disulfide are also known to be antibacterial (33,34) and display antifungal and antiviral activities (7,27).

Bacteria have evolved resistance to virtually all antibiotics introduced into clinical practice (11) leading to treatment failure of infections of both animals and humans. The purpose of the present study was to investigate the potential for TDA exposure to select for mutants resistant to TDA and other agents and to determine mechanisms of resistance to TDA.

MATERIALS AND METHODS

Bacterial strains and growth media. The strains used in this study are listed in Table 1. All strains were grown on LB agar (Oxoid CM1021, Basingstoke, UK) or in LB broth (Oxoid CM1018) at 37°C except for the strains *Phaeobacter* 27-4, *Phaeobacter* JBB1001 and *V. anguillarum* 90-11-287, which were cultured on marine agar 2216 (MA; Difco, BD, Sparks, MD) or in marine broth 2216 (MB; Difco) at 25°. Unless otherwise stated, all strains were incubated for one day except for *Phaeobacter* 27-4 which were incubated for two to three days. The strains were either stored on Protect^{TM} beads or in a freeze medium (30.0 g Tryptone Soya Broth (Oxoid CM129B), 5.0 g glucose, 20.0 g skimmed milk powder, 40.0 g glycerol, 1000 ml H_2O) at -80°C.

Purification of TDA. *Phaeobacter* 27-4 was grown in 1.5 l MB for four to five days at 25°C at stagnant conditions. The culture was extracted twice with an equal volume ethyl acetate containing 1 % formic acid and shaken for 10 min in a separation funnel. After phase separation the upper phase was filtered through anhydrous Na_2SO_4 (~100 g per L ethyl acetate) and evaporate in vacuo. Extract from 1.5 l culture was then redissolved in 10 ml 15% acetonitrile and loaded onto a 1 g Strata-XC column (Phenomenex, Torrance, CA) which was washed with 20 ml 15% acetonitrile and eluted with 10 ml 70% acetonitrile, which was evaporated using a stream of N_2.

Purity of TDA was verified by LC-UV-HR-MS analysis as described previously (5) as well as UHPLC-UV/VIS analysis which was perform formed on a RSLC Ultimate 3000 system (Dionex, Sunnyvale, CA) using a 150 mm × 2 mm i.d., 2.6 µm Kinetex C_{18} column (Phenomenex), running at 800 µl/min and 60°C using a binary linear solvent system of water (A) and acetonitrile (B) (both buffered with 50 µl/l trifluoroacetic acid). The gradient program was: t=0, 15% B; t= 0.5 min 25% B; t= 6 min 65% B, and t=7 100% B, keeping this for 1 minutes, then reverting to 15% in 1 min.

Antimicrobial activity of TDA. The minimum inhibitory concentration (MIC) of TDA against a range of bacteria were determined by the microbroth dilution method (1,2). Semi-purified TDA (see above) was diluted in 50% ethanol and two-fold dilutions of this TDA stock solution (2530 mg/l) prepared in microtiter plates. After inoculation with approximately 10^5 cfu/ml, the human pathogenic bacteria were incubated at 37°C for 24 h and the marine bacteria at 25°C for 2 days (Table 1). Growth was determined visually. All MIC determinations were repeated two times in independent experiments. To determine if porins or efflux pumps were involved in innate tolerance to TDA, MIC values of TDA against mutants of *Salmonella enterica* serovar Typhimurium (hereafter referred to as *S.* Typhimurium) with disruptions in the relevant genes (see Table 1) were

determined as described above. The MIC of TDA in agar was also determined for *S.* Typhimurium SL1344, *E. coli* NCTC 10538, *P. aeruginosa* NCTC 10662 and *Staphylococcus aureus* 12493. Two fold dilutions of TDA stock solution were incorporated into LB agar (1,2). Cultures were diluted and 10^4 cfu spotted onto the agar. Results (growth or no growth) were read after 24 incubation at 37°C.

Effect of TDA upon bacterial growth. To determine the killing kinetics of TDA, *S.* Typhimurium SL1344 and *S. aureus* NCTC 12493 were grown in LB at 37°C for one day (300 rpm) and inoculated to 10^6 cfu/ml in either LB or phosphate buffered saline (PBS) or LB or PBS containing TDA at concentrations of MIC, ½MIC or ¼MIC. The strains were incubated at 37°C (rpm 300) and numbers of bacteria determined by plate count at appropriate time points. The experiment was carried out in duplicate.

Selection of TDA resistant mutants. Four different experimental approaches were used to select TDA resistant mutants. All methods included a single exposure to TDA administered via different methods.

Exposure to 2xMIC of TDA on agar. Overnight cultures of *S.* Typhimurium SL1344, *E. coli* NCTC 10538, *P. aeruginosa* NCTC 10662 and *S. aureus* NCTC 12493 were diluted or concentrated to give a range of inocula of 10^5-10^9 cfu/ml (38). 100 µl of each suspension were spread onto LB agar containing 2x the MIC of TDA for each strain. Plates were checked every day for three days for appearance of TDA-resistant colonies. Colonies which arose were retained and TDA MIC values were determined.

Exposure to MIC or 2xMIC of TDA in broth. Cultures of all strains (apart from the *Salmonella* mutants with gene disruptions) were inoculated (10^7 and 10^8 cfu/ml) into liquid media

containing either MIC or 2xMIC of TDA in microtiter trays. After incubation for one (human pathogens) or two (marine strains) days, a drop of liquid culture was plated on LB or MA plates and any colonies arising were retained. The MIC of TDA against these colonies was determined as described above.

TDA diffusion mutant selection. Cultures of *S.* Typhimurium SL1344, *P. aeruginosa* NCTC 10662, *E. coli* NCTC 10538, *S. aureus* NCTC 12493 and *V. anguillarum* 90-11-287 were diluted in PBS to approximately 10^5, 10^6, 10^7 and 10^8 cfu/ml were plated onto agar plates. Paper disks or filter paper in different patterns were placed on the bacterial lawns and TDA stock solution (2500 mg/l) pipetted onto the disks or paper. Wells were also drilled into the agar and 70 µl of TDA stock solution was added. Plates were incubated for 1 (human pathogens) or 2 (*V. anguillarum* 90-11-287) days and observed for appearance of colonies within the clearing zone formed by diffusion of TDA into the agar. Colonies were isolated, retained and the MIC of TDA determined.

TDA resistant Tn5-mutants. A random transposon insertion library in *S.* Typhimurium SL1344 based on the Tn5 and Mu transposon (9) were screened for mutants with increased TDA tolerance. Approximately 10^4 insertion mutants were screened for the ability to grow in TDA (2xMIC) and kanamycin (25 mg/l). Culture grown without TDA served as a control. After incubation at 37°C for one day, growth was observed visually and cultures were plated onto LB agar plates.

Selection of TDA tolerant strains. Three approaches were used to select sub-populations of strains with elevated tolerance to TDA.

Exposure to ½MIC to 2xMIC of TDA. Cultures (Table 1 strains apart from the *Salmonella* mutants with gene disruptions) were inoculated (10^5 cfu/ml) into medium containing ½MIC of TDA and after incubation overnight 20 µl of the cultures were inoculated into fresh broth

containing the MIC of TDA (200 µl) incubated again and the following day 20 µl of culture was inoculated into fresh broth containing 2xMIC of TDA. Experiment was conducted in triplicates.

Exposure to ½MIC of TDA. *S.* Typhimurium SL1344, *P. aeruginosa* NCTC 10662, *E. coli* NCTC 10538, *S. aureus* NCTC 12493, *V. anguillarum* 90-11-287 and *Phaeobacter* 27-4 were re-inoculated (30 µl) six times in medium (2970 µl) containing ½MIC of TDA. These transfers equaled 42-48 generations. Human pathogenic bacteria were incubated for one day at 37ºC, whereas marine bacteria were incubated for two day at 25ºC.

Exposure to 1/16MIC to MIC of TDA. *S.* Typhimurium SL1344 was re-inoculated (10 µl) five times into 990 µl LB and then 40 times into gradually increasing concentrations of TDA (from 1/16MIC to MIC), which in total equals 315-360 generations. The experiment was carried out with triplicate lineages. A control in duplicate lineages was also re-inoculated (10 µl into 990 µl LB) 45 times (315-360 generations), but without TDA. All cultures were inoculated once per day and incubated at 37°C. At the end of all three adaptive approaches, MIC of TDA were determined both for cultures exposed to TDA but also for control cultures.

RESULTS

Antimicrobial activity of TDA. TDA inhibited the growth of both Gram negative and Gram positive bacteria in broth; the latter group (80 – 155 mg/l) was generally more susceptible to TDA than the Gram-negative bacteria (625 – 1250 mg/l) (Table 1). *V. anguillarum* 90-11-287, *Phaeobacter* 27-4 and *Morganella morganii* NCTC 235 were exceptions, as they were inhibited by concentrations similar to the Gram positive bacteria even though they are Gram negative. The solvent of TDA (50% ethanol) did not inhibit the bacteria in the dilutions used. TDA is degraded at high temperatures (5) so as a control, the values were also determined for TDA that had been

incubated at 37°C or 25°C prior to inoculation. This gave the same MIC values as when inoculating straight away (data not shown) and hence, degradation of TDA did not take place during our assay. None of the porin or efflux pump mutants of *S.* Typhimurium had major altered tolerance to TDA compared with their parental strains.

Effect of TDA upon bacterial growth. TDA is a bactericidal compound as both *S.* Typhimurium SL1344 and *S. aureus* NCTC 12493 were killed by the MIC value in both LB (Figure 1A and 1C) and in PBS (Figure 1B and 1D). The reduction in cell numbers was greater for the *S. aureus* (≥ log 5 reduction) compared with the Gram negative bacterium (log 3 reduction) in LB broth. At ½MIC and ¼MIC killing of both *S.* Typhimurium SL1344 and *S. aureus* NCTC 12493 in PBS was seen. A dose-response kinetic relationship was observed for both the growth media and the buffer. As the concentration of TDA increased, the faster the bacteria were killed or growth of the bacteria was inhibited for longer.

Selection of TDA resistant mutants. None of the experimental approaches used in this study resulted in stable TDA resistant mutants. Although *S.* Typhimurium SL1344, *E. coli* NCTC 10538, *P. aeruginosa* NCTC 10662 and *S. aureus* NCTC 12493 all gave rise to colonies on agar containing 2xMIC of TDA, the MIC of TDA was the same as prior to TDA exposure after one inoculation in fresh media. Independent of inoculation level (10^7 or 10^8 cfu/ml), none of the strains survived exposure to 2xMIC of TDA in broth (except *S. aureus* NCTC 8532). When inoculating in broth containing the MIC of TDA, 10 of 18 strains survived (*S.* Typhimurium SL1344, ATCC 14028s and L3, *Enterobacter cloacae* NCTC 10005, *Serratia marcescens* NCTC 2847, *P. aeruginosa* NCTC 10662, *Klebsiella pneumonia* NCTC 10896, *M. morganii* NCTC 235, *S. aureus* NCTC 8532, *Enterococcus faecalis* NCTC 775 and NCTC 7171 and *Listeria monocytogenes* NC12427).

However, after one passage in fresh medium, no altered MIC values were observed when compared to the original strains. In experiments using diffusion of TDA in to agar, no colonies appeared inside the clearing zones for any of the five strains tested in any of the three diffusion methods used. A transposon insertion library in *S.* Typhimurium SL1344 was exposed to 2xMIC in liquid medium and plated on agar plates. However, no colonies with decreased susceptibility to TDA appeared.

Selection of TDA tolerant strains. Two of the three adaptation experiments resulted in "variants" that tolerated slightly elevated TDA concentrations. However, the phenotype was not stable after passage in drug free media. In the first adaptation experiment, concentrations of TDA were increased from ½MIC to MIC and to 2xMIC, nine of the 18 strains (*S.* Typhimurium L10, *E. cloacae* NCTC 10005, *S. marcescens* NCTC 2847, *P. aeruginosa* NCTC 10662, *K. pneumonia* NCTC 10896 and NCTC 9633, *E. coli* NCTC 10538, *V. anguillarum* 90-11-287 and *L. monocytogenes* NC12427) were able to survive and grow on 2x the MIC of TDA. However, when re-tested none of these nine strains had decreased susceptibility to TDA. In the second adaptation experiment, where *S.* Typhimurium SL1344, *P. aeruginosa* NCTC 10662, *E. coli* NCTC 10538, *S. aureus* NCTC 12493, *V. anguillarum* 90-11-287 and *Phaeobacter* 27-4 were exposed to six re-inoculations in ½MIC of TDA, the MIC of TDA required to inhibit the final populations were two-fold higher than for parental strains. However, after one passage in fresh medium none of the strains had altered susceptibility to TDA. In the last experiment, *S.* Typhimurium SL1344 was exposed to gradually increasing concentrations of TDA for a prolonged period (40 transfers). The MIC of TDA was two-fold higher for the final cultures arising in the experiment (all three lineages). However, after one inoculation in fresh medium, the MIC again reverted to the original wild-type level.

DISCUSSION

It has been previously shown that TDA producing *Roseobacter* have the ability to inhibit or kill other marine bacteria (3,13,22,42), and that purified TDA alone has a growth inhibitory effect against marine bacteria (3). This study shows that pure TDA is a strong antibacterial compound, effective against all the human pathogenic strains tested including strains resistant to conventional antibiotics. The tautomeric form of TDA, thiotropocin, produced by *Caulobacter* and *Pseudomonas* strains also inhibited the growth of all tested bacteria (27,49). Both Gram negative and –positive human pathogens were affected by TDA, however, Gram positive bacteria generally required a lower concentration for inhibition compared to Gram negatives. This could be due to the different structures of the cell wall in the two bacterial groups, and the outer membrane in the Gram negative bacteria could act as a protective barrier against TDA. Treatment of the Gram negative strain *S.* Typhimurium SL1344 with 0.5 mM EDTA (this concentration does not affect growth) rendered the cells more sensitive to TDA, whereas this is not the case for the Gram positive strain *S. aureus* NCTC 12493 (data not shown). These data support the hypothesis that the outer membrane of the cell envelope gives protection to Gram negative bacteria, as EDTA is known to destabilize this membrane by binding divalent cations (29).

Neither the porin mutants nor efflux pump mutants of *S.* Typhimurium had elevated tolerance towards TDA compared to their parental strains. This indicates that perhaps TDA is neither transported into the bacterial cell through porins nor exported out again through the major efflux pumps. Keeping in mind that TDA works very well on non-growing cells these results could collectively indicate that TDA may not have to enter the bacterial cell in order to exert its effect. Thereby the cell envelope could be the bacterial target for TDA. This theory is supported by results from a biosensor method, which is based on activation of fluorescence signals (GFP variants) engineered to report induction of genes responsive to inhibition or perturbation of various core

metabolic pathways of *Salmonella*. This method showed that the cell envelope is damaged by TDA and the peptidoglycan structure might be directly affected (Dirk Bumann, University of Basel, personal communication).

V. anguillarum 90-11-287 was very sensitive to TDA and inhibited by a MIC value in the same range as the Gram positive bacteria. The producer strain of TDA (*Phaeobacter* 27-4) was isolated specifically due to its antagonistic effect against this strain (22). The Gram negative TDA producer strain itself is also inhibited by a low TDA concentration and did not, in our assay, display the expected resistance to TDA. It is possible that these data were obtained as the conditions used in the MIC value determination with only two days of incubation did not support production of TDA by *Phaeobacter* 27-4. TDA is produced in late exponential phase and requires a large surface-volume ratio (4,5). We hypothesize that only when TDA is produced does the producer organism express the mechanism(s) by which it become(s) resistant to its own drug.

TDA was bactericidal against both *S.* Typhimurium SL1344 and *S. aureus* 12493, and the MIC value for each strain was also lethal in rich growth medium (LB). This indicates that the MIC and the minimum bactericidal concentration (MBC) are the same for TDA as has been reported for some host defense peptides (18). Bacteria treated with TDA were unable to multiply, but remained viable for one hour after which the bacteria died. The same phenomenon has been described for the host defense peptide Plectacin (45). When carrying out killing kinetics in a nutrient poor medium (PBS), where essentially no cell division occurs, all tested concentrations of TDA kill the bacteria rapidly. These results suggest that TDA is active against both growing and non-growing cells. This has been reported for glycan-targeting antibiotics like moenomycin and teicoplanin (10,50).

Several different methods for selecting TDA resistant mutants or strains with tolerance towards TDA were explored in this study. Most of the methods reported in the literature

for such a purpose were used. We did not use the gradient gel method (10), as this technique requires large volumes of the compound of interest and our available, pure supply of TDA was limited.

None of the methods of single exposure to TDA resulted in mutants resistant to TDA. This could indicate that more than one mutation is needed in order to gain resistance. In this manner, TDA may resemble 'dirty drugs' such as biocides or antimicrobial peptides that have multiple targets in bacteria and thus require mutations in multiple loci in order to evolve tolerance (37,43), although biocides are often mixtures and TDA is not. A slow adaptation to gradually increasing concentrations of the drug has proven to be a successful way of getting stable strains with enhanced tolerance to an antimicrobial peptide and to biocides (24,32,36,48) but a similar approach was not successful in this study as *S.* Typhimurium SL1344 immediately lost the gained tolerance. Earlier studies showed a gradual deadaptation of the decreased sensitivity to different biocides (23,32,44,48) however Sallun & Chen (2008) reported that the tolerance toward the antimicrobial peptide Cecropin B was abolished after one passage in fresh medium without the peptide (44) just as found in the present study with TDA.

Tolerance to a drug can, apart from 'fixed' mutation, also occur as a response to environmental stimuli leading to a phenotypic response (30). It is doubtful that true adaptation to TDA was seen in this study, as the tolerance was only two fold higher greater than non-adapted cells, whereas in the literature for quaternary ammonium compounds and the antimicrobial peptide cecropin it is reported as many fold higher (23,32,44). The increased tolerance to TDA seen in this study could be the result of a phenotypic switch which is rapidly reversed post exposure to TDA.

Based on the results obtained in this study, we conclude that TDA has a wide spectrum of activity against pathogenic bacteria. We also conclude that it is difficult to create stable strains with tolerance to TDA. We suggest that the compound resembles antimicrobial

peptides and biocides with regard to mechanism-of-action as compared to antibiotics. As only a slight increase in TDA tolerance was observed and any TDA tolerance phenotype was unstable TDA is an interesting compound for the potential control of bacterial diseases in aquaculture as well as in other settings.

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Legends to figures

Figure 1. Killing kinetics of tropodithietic acid (TDA) against *Salmonella enterica* serovar *Typhimurium* strain SL1344 (A and B) and *Staphylococcus aureus* NCTC 12493 (C and D) in LB medium (A and C) and in phosphate buffered saline (B and D). The following concentrations of TDA were used: MIC (◇), ½MIC (□), ¼MIC (△) and a growth control containing no TDA (○). Counts are mean of duplicates and error bars represent standard deviations. Vertical arrows indicate detection limit.

Table 1 Bacterial strains used in this study.

Gram	Species	Strain	Genotype	References / Sources
Negative	*Salmonella* Typhimurium	SL1344	Wildtype	Wray & Sojka, 1978 (51)
	Salmonella Typhimurium	SL1344	*ompF*::*aph*	Coldham et al., 2010 (12)
	Salmonella Typhimurium	SL1344	*ompC*::*aph*	Coldham et al., 2010 (12)
	Salmonella Typhimurium	ATCC 14028s	Wildtype	ATCC [a]
	Salmonella Typhimurium	ATCC 14028s	Δ*tolC::Cm*^{R}	Nishino et al., 2006 (35)
	Salmonella Typhimurium	ATCC 14028s	Δ*acrB::Km*^{R}	Nishino et al., 2006 (35)
	Salmonella Typhimurium	ATCC 14028s	Δ*acrAB::Cm*^{R}	Nishino et al., 2006 (35)
	Salmonella Typhimurium	ATCC 14028s	Δ*acrD::Cm*^{R}	Nishino et al., 2006 (35)
	Salmonella Typhimurium	ATCC 14028s	Δ*acrEF::Cm*^{R}	Nishino et al., 2006 (35)
	Salmonella Typhimurium	ATCC 14028s	Δ*mdtABC::Cm*^{R}	Nishino et al., 2006 (35)
	Salmonella Typhimurium	ATCC 14028s	Δ*mdsABC::Cm*^{R}	Nishino et al., 2006 (35)
	Salmonella Typhimurium	ATCC 14028s	Δ*ermAB::Cm*^{R}	Nishino et al., 2006 (35)
	Salmonella Typhimurium	ATCC 14028s	Δ*mdfA::Cm*^{R}	Nishino et al., 2006 (35)
	Salmonella Typhimurium	ATCC 14028s	Δ*mdtK::Cm*^{R}	Nishino et al., 2006 (35)
	Salmonella Typhimurium	ATCC 14028s	Δ*macAB::Cm*^{R}	Nishino et al., 2006 (35)
	Salmonella Typhimurium	L3	Human pre-therapy clinical isolate	Piddock et al., 2000 (39)
	Salmonella Typhimurium	L10	Human post-therapy clinical isolate, *acr*AB+++	Piddock et al., 2000 (39)
	Enterobacter cloacae	NCTC 10005		NCTC [b]
	Serratia marcescens	NCTC 2847		NCTC [b]
	Klebsiella pneumoniae	NCTC 10896		NCTC [b]
	Klebsiella pneumoniae	NCTC 9633		NCTC [b]
	Escherichia coli	NCTC 10538, K12		NCTC [b]
	Morganella morganii	NCTC 235		NCTC [b]
	Pseudomonas aeruginosa	NCTC 10662		NCTC [b]
	Vibrio anguillarum	90-11-287 (from rainbow trout)		Skov et al., 1995 (47)
	Phaeobacter ssp.	27-4 (from Spanish turbot unit)	Wildtype	Hjelm et al., 2004 (22)
	Phaeobacter ssp.	JBB1001	*tdaB::*EZ-Tn5,Kan	Geng et al., 2008 (16)
Positive	*Staphylococcus aureus*	NCTC 8532 (MSSA)		NCTC [b]
	Staphylococcus aureus	NCTC 12493 (MRSA)		NCTC [b]
	Enterococcus faecalis	NCTC 775		NCTC [b]
	Enterococcus faecalis	NCTC 7171		NCTC [b]
	Listeria monocytogenes	NC12427		NCTC [b]

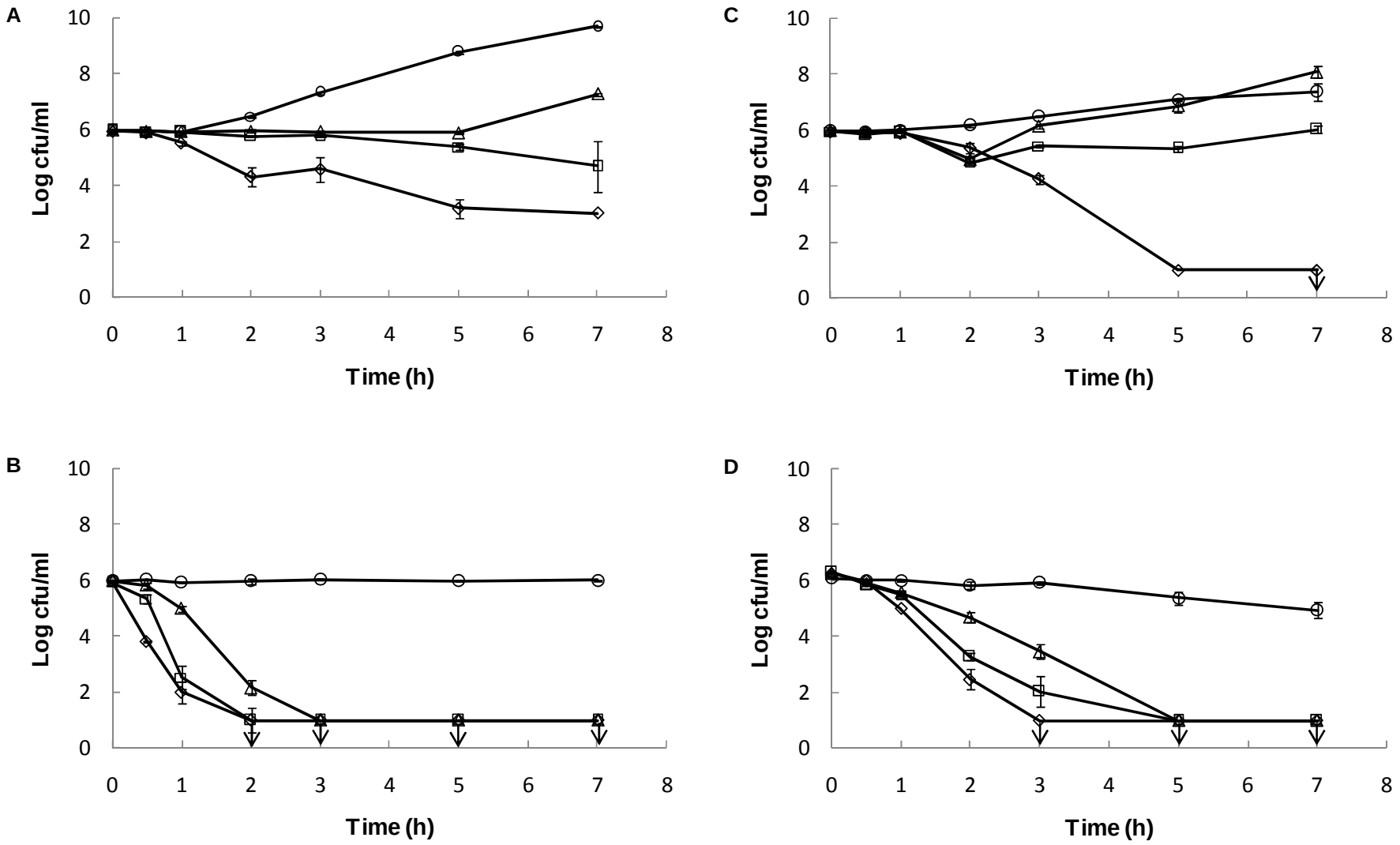
[a] ATCC: American Type Culture Collection

[b] NCTC: National Collection of Type Cultures (HPA, Colindale, London, UK)

Table 2 MIC values (mg/l) of tropodithietic acid determined in LB broth. MICs were determined in two independent experiments and if all gave the same value, this is stated in the table. For some strains, MIC varied a factor of two between experiments as indicated.

Gram	Species	Strain	MIC (mg/l)
Negative	*Salmonella* Typhimurium	SL1344 (wildtype)	625
	Salmonella Typhimurium	SL1344 (*ompF*::*aph*)	625
	Salmonella Typhimurium	SL1344 (*ompC*::*aph*)	625
	Salmonella Typhimurium	ATCC 14028s (wildtype)	625
	Salmonella Typhimurium	ATCC 14028s (Δ*tolC::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*acrB::Km*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*acrAB::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*acrD::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*acrEF::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*mdtABC::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*mdsABC::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*ermAB::Cm*^{R})	625
	Salmonella Typhimurium	ATCC 14028s (Δ*mdfA::Cm*^{R})	625
	Salmonella Typhimurium	ATCC 14028s (Δ*mdtK::Cm*^{R})	625 - 1250
	Salmonella Typhimurium	ATCC 14028s (Δ*macAB::Cm*^{R})	625
	Salmonella Typhimurium	L3 (Human pre-therapy clinical isolate)	625 - 1250
	Salmonella Typhimurium	L10 (Human post-therapy clinical isolate, *acr*AB+++)	625 - 1250
	Enterobacter cloacae	NCTC 10005	625 - 1250
	Serratia marcescens	NCTC 2847	625 - 1250
	Klebsiella pneumoniae	NCTC 10896	625 - 1250
	Klebsiella pneumoniae	NCTC 9633	625
	Escherichia coli	NCTC 10538 (K12)	155 - 625
	Morganella morganii	NCTC 235	155
	Pseudomonas aeruginosa	NCTC 10662	625
	Vibrio anguillarum	90-11-287 (from rainbow trout)	40 - 80
	Phaeobacter ssp.	27-4 (from Spanish turbot unit) (wildtype)	310
	Phaeobacter ssp.	JBB1001 (*tdaB::*EZ-Tn5,Kan)	155
Positive	*Staphylococcus aureus*	NCTC 8532 (MSSA)	155
	Staphylococcus aureus	NCTC 12493 (MRSA)	155
	Enterococcus faecalis	NCTC 775	80
	Enterococcus faecalis	NCTC 7171	80
	Listeria monocytogenes	NC12427	80

1



2 Figure 1